METABOLIC INTERRELATIONS

WITH SPECIAL REFERENCE TO

CALCIUM

Transactions of the Fifth Conference

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1—Shorr 2—McLean 3—Reifenstein 4—Albright 5—Armstrong 6—Howard 7—Gutman 8—Bartter 9—Engel 10—Sobel 11—Butler 12—Stearns 13—Fremont Smith 14—Kramer 15—Henneman 16—Follis 17—Rubin 18—Stevenson 19—McCance 20—Barnett 21—Neuman 22—Park 23—Hodge 24—Copp 25—Urist 26—Robinson 27—Handler and 28—Harrison

JOSIAH MACY JR FOUNDATION CONFERENCE PROGRAM

FRANK FREMONT SMITH
Medical Director

As AN INTRODUCTION to these TRANSACTIONS of the Fifth Conference on Metabolic Interrelations I should like to outline what it is that the Foundation hopes to accomplish by its Conference Program We are interested first of all in furthering knowledge about Metabolic Interrelations and to this end the participants were brought together to exchange ideas experiences data and methods In addition to this particular goal however there is a further and perhaps more fundamental aim which is shared by all our conference groups This is the promotion of meaningful communication between scientific disciplines

The problem of communication between disciplines we feel to be a very real and urgent one the most effective advancement of the whole of science being to a large extent dependent upon it Because of the accelerating rate at which new knowledge is accumulating and because discoveries in one field so often result from information gained in quite another channels must be established for the most effective dissemination and exchange of this knowledge

The increasing realization that nature itself recognizes no boundaries makes it evident that the continued isolation of the several branches of science is a serious obstacle to scientific progress Particularly is it true in medicine that the limited view through the lens of one discipline is no longer enough For example today medicine must be well versed in nuclear physics because of the tracer techniques and the injury which can result from radiation At the other extreme medicine is certainly a social science and through mental health must be concerned with economic and social questions The answer then is not further fragmentation into increasingly isolated specialties disciplines and departments but the integration of science and scientific knowledge for the enrichment of all branches This integration we feel can be encouraged by providing opportunities for a multiprofessional approach to given topic

Although the fertility of the multiprofessional approach is recognized adequate provision is not made for it by our universities scientific societies or journals And perhaps the presence of other hindering factors must be admitted Partly semantic in nature they may also to some degree be psychological Admittedly it is oftentimes difficult to accept data derived from methods with which one is unfamiliar By making free and informal discussion the central core of our meetings we hope to achieve an atmosphere which minimizes as much as possible the semantic and emotional barriers

that within certain rather fixed limits the serum has the capacity to carry much more calcium than it normally does without precipitation of calcium phosphate over rather wide periods of time The ultrafilterable fraction of calcium rises but so does the quantity of calcium present as proteinate (non ultrafilterable) in an extraordinarily fixed proportion until a total concentration of 20 mg of calcium per 100 cc of serum is reached This is shown in Table I from Dr Hopkins work which has been repeated over and over again When one progressively raises the serum calcium level the filterable calcium rises and the serum phosphorus remains the same until one gets to the critical point of 20 mg At this point the percentage of filterable calcium falls off sharply and the serum phosphorus percentage falls off also This conclusion is based on about twenty experiments

Shorr And how is this determined—*in vivo* or *in vitro*?

Howard We have not studied this problem *in vivo* up to the present because I thought the procedure was potentially dangerous It reminded us of acute hyperparathyroidism where the homeostatic mechanisms suddenly fail We have had opportunity to do two sera over 20 with the phosphorus level reasonably normal and in both of them this same phenomenon held good However the patients died quite soon afterward

Shorr In what form is the calcium added?

Howard As three or four different kinds of calcium salts other than phosphate

Armstrong One other question In the last two lines of the data of Table I at 19 4 mg serum calcium the filterable calcium is 13 5 mg but when the total calcium is raised slightly to 20 3 the filterable calcium drops to 11 Did the total quantity of filterable calcium decrease from 13 5 to 11 simply by increasing the total serum calcium content?

Sobel The trouble is that this was a supersaturated solution

Howard Well that depends on the definition of supersaturation This relationship will hold good for a long time

Armstrong This is more than a percentage change It is an absolute change isn t it?

Howard That is right I think that what is held back is entirely different as I shall come to in a minute I will give you our thesis anyway

Sobel How many hours did you wait before filtration?

Howard We have added calcium to serum and waited as long as six

weeks Dr Hastings brought up that question We kept serum frozen for six weeks and found no difference

At and beyond the total concentration of 20 mg of calcium per 100 cc of serum the ultrafilterable serum phosphorus falls off sharply and the ultrafilterable calcium component may or may not be reduced coincidentally Something has happened a critical point has been reached at which the normal relationships of these serum components have been altered For want of a better theory we have assumed the production of a protein calcium complex which is non diffusible But no visible precipitate forms and nothing appears on centrifugation of such sera

THE RELATIONSHIPS IN ABNORMAL SERUM

When one starts with a serum higher than normal in *phosphorus* content (artificially produced or pathological sera such as from patients with renal insufficiency) the previously mentioned changes in ultrafiltration appear at lower concentrations of calcium *but still always above the normal* unless one reaches phosphorus levels of 15 mg per 100 cc of serum or more (Tables II and III) We have presumed that when concentrations of calcium and phosphorus are reached *in vivo* such as to yield altered ultrafiltration figures *in vitro* such complexes act as foreign bodies and are either precipitated or engulfed by clasmatocytes as shown in the experiments of Gersh

TABLE II

The Effect of an Increased Serum Calcium Level in the Presence of a High Serum Phosphorus Level on the Ultrafilterable Calcium and Phosphorus

(Total Protein = 6 7 gm./100 cc)

No	Filtrate pH	Calcium			Phosphorus		
		Serum	Filtrate		Serum	Filtrate	
		(mg./100 cc)	(mg./100 cc)	(%)	(mg./100 cc)	(mg./100 cc)	(%)
30A	7 62	10 4	6 5	63	9 7	10 1	104
30B	7 52	12 7	8 2	65	9 8	9 8	100
30C	7 43	15 2	8 7	57	9 8	8 9	91
30D	7 34	17 6	10 6	60	9 8	8 3	85

Gersh I Histochemical Studies on the Fate of Colloidal Calcium Phosphate in the Rat *Anat Rec* 70 331 (1938)

b Gersh I The Fate of Colloidal Calcium Phosphate in the Dog *Am. J Physiol* 121 589 (1938)

TABLE III

The Effect of an Increased Serum Calcium Level in the Presence of a High Serum Phosphorus Level on the Ultrafilterable Calcium and Phosphorus

(Total Protein = 6.5 gm./100 cc.)

No.	Filtrate pH	Calcium			Phosphorus		
		Serum	Filtrate		Serum	Filtrate	
		(mg./100 cc.)	(mg./100 cc.)	(%)	(mg./100 cc.)	(mg./100 cc.)	(%)
31E	7.42	10.4	5.5	53	15.6	16.0	103
31A	7.42	11.0	6.0	55	14.6	14.4	99
31B	7.48	13.6	4.5	33	14.6	13.5	92
31C	7.42	15.6	6.3	40	14.7	12.3	84
31D	7.38	18.3	6.4	35	14.8	10.9	74

But perhaps more important than this to the clinician is the concept gained from these experiments that normal serum is *not saturated* as to calcium—at least, if either more calcium or phosphorus is added to the serum by some pathological process, there is no situation in the serum itself which requires the phosphorus concentration to fall when the calcium rises or the calcium to fall when the phosphorus rises. If such reciprocal changes in concentration *do occur*, they are then *the result of physiological mechanisms* and hence induced by cellular changes or at least by activity somewhere in the cellular compartment as distinguished from the extracellular. As a matter of fact, as will be pointed out later, the serum phosphorus level rises when the serum calcium concentration is raised by the injection of calcium salts.

Before going into the factors which we believe play roles in calcium homeostasis, perhaps it would be well to mention a recent observation of Dr. Yendt[1] which seems pertinent to our concepts of the calcium-phosphorus relationships at the extracellular level. In carrying out some experiments with *in vitro* calcification of rachitic cartilage by ultrafiltrates from various normal and pathological sera, Dr. Yendt rewarmed the ultrafiltrates to body temperature in an incubator in unstoppered (cotton plugged) bottles. To his amazement, within a few minutes, all of the ultrafiltrates

[1] Yendt, E. R. Unpublished observations.

became cloudy and a precipitate soon formed It was found that the pH of such ultrafiltrates was 8 5 If before heating to body temperature the ultrafiltrate was saturated with 5 per cent CO in O so that the pH thus (presumably) was lowered to 7 5 and then the bottle was corked no such precipitate formed and the experiment could be carried out as planned [1]

Incidentally the ultrafiltrates of those sera which themselves will calcify rachitic cartilage have thus far also caused the cartilage to calcify [a] I might mention that these ultrafiltrates contain no protein by ordinary tests and their nitrogen content closely approximate that of the serum non protein nitrogen [b] Furthermore the whole serum under such conditions (i e incubated to body temperature and shaken) may reach a pH of 9 3 or higher from the loss of CO and a small precipitate form but certainly it usually does not form a precipitate or else the classical experiments of Shipley Kramer and Howland could not have been performed

Dr Park tells me—I see Dr Kramer is here now—that in their experiments they did not use stoppered bottles but did run the test in an incubator Is that right?

Kramer We did use stoppered bottles

Howard You did? We searched thoroughly in all of your articles and could find no reference to the use of a stopper

Park Do you know whether Shipley used stoppered bottles?

Kramer I do not know what he used but I used them

Howard Since we have been visualizing the interstitial fluid as essentially a plasma ultrafiltrate one would have to believe from this that from normal interstitial fluid calcium phosphate will soon precipitate wherever a pH of 8 5 is extant

The Homeostasis of Calcium in Body Fluids

Now since serum and interstitial fluid calcium are in dynamic equilibrium (in the bones—probably a changing one from one end of the capillary to

---

[1] Dr Yendt found also that if the pH of the ultrafiltrate is kept at 7 5 by passing 5 per cent CO through it calcium can be added to the filtrate up to a concentration greater than 20 mg per 100 cc even in the incubator without visible precipitation

a Shipley P G Kramer B and Howland J Calcification of Rachitic Bones In Vitro Am J Dis Child 30 37 (1925)
b Shipley P G Kramer B and Howland J Studies upon Calcification In Vitro Biochem J 20 379 (1926)

the other (*vide infra*) we may turn to the question of what governs the extraordinary stability of the serum calcium concentration which McLean and Hastings[3] have aptly called "one of nature's physiological constants." I need not cite to this audience the markedly unphysiological conditions an experimenter must impose upon his subject in order to obtain appreciable deviations of the serum calcium level. An animal can be subjected to an overall negative calcium balance by several means—deprivation of dietary calcium,[14] lactation coincident with inadequate intake,[15] vitamin D deficiency[16] (where absorption is presumably interfered with)—all for prolonged periods without producing appreciable hypocalcemia. One may even repeatedly bleed and retransfuse with calcium depleted blood and obtain only transitory hypocalcemia with quick restoration to normal levels.[17] The opposite situation is equally well taken care of: intravenous loads up to 1 gram of calcium given in four hours are quickly disposed of by normal persons with a rapid return to normocalcemia with only 30 to 50 per cent of the calcium introduced appearing in the urine within 24 hours.[18, 19]

14a Boelter, M. D. D., and Greenberg, D. M.: Severe Calcium Deficiency in Growing Rats. II. Changes in Chemical Composition. *J. Nutrition* 21: 75 (1941).
b Kramer, B., and Howland, J.: Factors which Determine the Concentration of Calcium and of Inorganic Phosphorus in the Blood Serum of Rats. *Bull. Johns Hopkins Hosp.* 33: 313 (1922).

15Liu, S. R., Chu, S. I., Su, C. C., Yu, T. T., and Cheng, T. Y.: Calcium and Phosphorus Metabolism in Osteomalacia. IX. Metabolic Behavior of Infants Fed on Breast Milk from Mothers Showing Various States of Vitamin D Nutrition. *J. Clin. Investigation* 19: 327 (1940).

16a Bauer, W., Marble, A., and Claflin, D.: Studies on the Mode of Action of Irradiated Ergosterol. Its Effect on the Calcium, Phosphorus and Nitrogen Metabolism of Normal Individual. *J. Clin. Investigation* 11: 1 (1932).
b Nicolaysen, R. V.: Studies upon the Mode of Action of Vitamin D. Influence of Vitamin D on the Absorption of Calcium and Phosphorus in the Rat. *Biochem. J.* 31: 122 (1937).

17Hastings, A. B., and Huggins, C. B.: Studies on the Effect of Alterations in the Concentration of Calcium in Circulating Fluids on the Mobilization of Calcium. *TRANS. MACY CONFERENCE ON METABOLIC INTERRELATIONS* 3: 38 (1951).

18Baylor, C. H., Van Alstine, H. E., Keutmann, E. H., and Bassett, S. H.: The Fate of Intravenously Administered Calcium. Effect of Urinary Calcium and Phosphorus, Fecal Calcium and Calcium Phosphorus Balance. *J. Clin. Investigation* 29: 1167 (1950).

19a Howard, J. E., Hopkins, T. R., and Connor, T. B.: The Use of Intravenous Calcium as a Measure of Activity of the Parathyroid Glands. *Trans. Assoc. Am. Physicians* 65: 351 (1952).
b Howard, J. E., Hopkins, T. R., and Connor, T. B.: On Certain Physiologic Responses to Intravenous Injection of Calcium Salts into Normal, Hyperparathyroid and Hypoparathyroid Persons. *J. Clin. Endocrinol. and Metab.* 13: 1 (1953).

BONE AS A SOURCE OF CALCIUM

The bones are tl e only po b e source for any sucl q ant t es of al c um as are re qu red by the more drast c w tl drawal exper n ents s ch as la tat on or Hast ngs b eed ngs T s es other tl an tl bones cart lage and teeth conta but very small quant t es of calc um [2] Whetl er or not o l er t ssues take u p calc un under the art fi ally nduced h percalce n a s not kno vn l ut t seems clear tl at the skeleton has a n ecl an n to pro de alc n un ler le r at on stress d t seen altogetl er l kely that tl e an e tru t re a ce t tl e l lk of tl e crem ent dur g the exper ts of a ndan e There are of co e pathologcal c rc m tances l cl w tl drawal and perhaps al dance are ot n et v th such mmed ate d helpful buffer g respo es tl ese w l be n e t oned later and tl o t loul t an p fie l n tl e d scus o of abno mal t es n calc um etabol sm

But fron purel quant tat ve and s n p le da a tl e concept seen s escap al le tl at the skeleto add t on to ts fu ct of prov l ng str ctural r g l t *tl e cl f factor n stab l z ng tl e calc m oncentrat on of tl e* trace lular fl l Un l r ary ng o l t ons n norm al persons tl en tl fl l p s ng o er tl e m croscop c keletal surfaces e ther can lo or take u cal un n ole u es And t tl e d nam c relat o sl p between tl e fl l a l tl e sk letal surfaces wh cl ul ately sets tl e le el of tl e ser cal m

TI E CALCIUM RESERVE

He dr ks[2] argue l n en l er of tl s gro p two y ars ago l v l s for *ulat of tl e e te t of tl e skeletal urfaces a a l l e for eq l l rat o*

M he l a l l s c o ke s found t of a o a 11 6 g an s of calc um n he bod of a l hy 3 yea l l man we gh g k og m 99 p n that al l u 1? g am — w e n h bones a d ee The k n co a ned f ve of the rema n ng l ams of cal um Blood and ext a ellu flu ds o a n app x ma v 900 mg of c m T e cal um w ues o her l an e ske s oug t to be a mos en e l e m and ha d y a a ab e fo ready m vem nt to l e exte o
Inc d n a h re an e or n p ng o cal u a on n the report by M hell *al* f the abu a ed data p o l mo e ca m n he bon s nd eeth an was fou l n he to al cada e

M e H H Ham on T S S egge da F P a d B a H W Tl e Chem ical C po x of e Adu Hu a B d an l I Le r g o B o em y of *C* ow h *J l C* 1a8 6 5 (1945

Howa l J E S m Tl ou o Calc um M ab l m a d B e Ph l gy *B ll f al M l T* 25 144 (1 5

Scot C H Tl e Local a of M ne al Sa C l of S *me Mamm l an* T u h M c o In era *A J* *Im* 53 43 (1933)

H l k S P an l H l W l Tl e N u e of P ne and Pl p e Rock *TRANS M ACY C NI EREN CE ON METABOLIC INTERRELATIONS* 3 173 (1951

with the body fluids and with the notion of the *quantity* of calcium particles attached to these *surfaces* but not part and parcel of the apatite lattice and hence readily available to what one might call passing influences When pressed for an estimate of the amount of such quickly available calcium Dr Hendricks made a guess of 100 grams or more [25] According to this concept there would be a tremendous reserve of calcium which could be yielded if the concentration in the fluids passing the surfaces was too low or accepted if too high without participation by breakdown or build up of the matrix held apatite at all The homeostasis of the serum calcium level under ordinary conditions would be set by these calcium particles loosely attached to the skeletal apatite In the presence of normal bone metabolism then factors operating at the skeletal surfaces will be the *major determinants of the level of the serum calcium* concentration

It is of considerable interest and importance we believe that when Hastings and Huggins repeated their calcium withdrawal experiment (frequent bleeding and replacement with calcium depleted blood) using parathyroidectomized dogs the bones were able to yield *just as much calcium* as was the case in the normal dogs *but* the calcium was yielded only at a lower level of serum calcium Thus hypoparathyroidism changed only the *level to which calcium had to fall before the skeleton began to render support* One could visualize that a change in pH of the fluids would influence the movement of these loose calcium particles—acidosis pulling them off alkalosis tending to push more on—and perhaps the level of inorganic phosphorus might have similar influences a fall in phosphorus creating a pull and a rise creating a push [26]

At any rate it seems to us that the evidence points strongly to the skeletal surfaces as being the biggest operators in the mechanism of stabilizing the concentration of calcium in the serum—be this normal or high or low Just how the parathyroid hormone may accomplish its action on this mechanism I do not know but certain it is that hyperparathyroidism *can* cause hypercalcemia for many years without any roentgenographic or microscopic evidence of *increased bone destruction* ([illegible] without elevation of serum alkaline pho phat[illegible] might indica[illegible] bone formation)

Only two other thoughts will be mentioned before leaving this matter of calcium homeostasis 1) If one starts with a perfectly normal organism and puts it in a state of negative calcium balance—for example by dietary calcium deficiency—at some stage a point must be reached at which Hendricks calcium particles are used up and the matrix apatite is called upon for further loss of calcium from the skeleton 2) There must be situations in which the number of Hendricks calcium particles is very few or at least the availability of such particles for support of calcium homeostasis is reduced Vitamin D appears to have something to do with such availability Rats after three weeks on a Steenbock rachitogenic diet without vitamin D exhibited a rapid fall in serum calcium and rise in phosphorus when subjected to starvation Control rats on the same diet but exposed to irradiations with the mercury vapor quartz lamp and deriving vitamin D in that manner when starved showed but little or no fall in serum calcium and smaller rises in phosphorus In humans diarrhea sometimes results in hypocalcemic tetany at an early date even with bones which by x ray appear normal yet in other patients similar degrees of diarrhea may be accompanied by normocalcemia even with extreme skeletal rarefaction as judged by roentgenogram

Factors Affecting the Calcium Balance

The system as a whole is affected of course by other factors and the interstitial fluid calcium content depends ultimately on how much enters and how much departs from it by all routes

THE GASTROINTESTINAL TRACT AND CALCIUM HOMEOSTASIS

The intestinal tract can be a pathway of both ingress and egress of calcium Our knowledge of calcium absorption by the gut is rather meager It seems quite certain that a high protein diet (if absorbed) and acidification of the upper tract tend to enhance calcium absorption [a, b] excess in the diet of either phosphorus or oxalate appears to retard calcium absorp-

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Harrison F Unpublished data (furnished by Dr E A Park)

Howard J E Some Current Concepts on the Mechanism of Calcification *J Bone Joint Surg* 33 A 801 (1951)

McCance R A Widdowson E M and Lehmann H The Effect of Protein Intake on the Absorption of Calcium and Magnesium *Biochem J* 36 686 (194[illegible])

a Telfer S V Calcium and Phosphorus Metabolism I The Excretion of Calcium and Phosphorus *Quart J Med* 16 45 (192[illegible])
b Telfer S V Studies in Calcium and Phosphorus Metabolism III The Absorption of Calcium and Phosphorus and Their Fixation in the Skeleton *Quart J Med* 17 [illegible]45 (19[illegible]4)

tion[3] [31d] Vitamin D seems a necessary adjuvant for maximal calcium absorption and deficiency of D greatly reduces it[16]

But it appears that in the *normal adult* at least the overall positive balance of calcium along the entire intestinal tract is very small[29] This conclusion is based upon the following reasoning we assume that our normal adult is in general calcium balance that his bones though having an active turnover are neither getting larger nor smaller The only path of egress of calcium other than in the stool is in the urine and rarely does one see more than 200 mg of calcium appearing in the 24 hour urinary output Therefore the normal positive calcium balance from the gut is no more than 200 mg per day usually about 100 mg per day There is abundant intestinal secretion (Gamble has estimated 8 litres per day)[32] from gastric and succus pancreatic juices and bile and if this is essentially a plasma transudate it would contain 400 mg to 560 mg of calcium depending on how one guesses the calcium concentration of plasma ultrafiltrate (*vide supra*) This calcium would get mixed with the ingested calcium forming a pool of let us say 1500 mg of calcium in a man on a 1000 mg calcium diet Of this total 1500 mg of calcium 600 is absorbed 100 appears in the urine and overall balance is maintained (Figure 1)

It has seemed to us remarkable how relatively *little* one alters the 24 hour urinary calcium by radical changes in calcium content of the diet The studies carried out on normals by Knapp[33] showed but small reflections of dietary changes in the urine (approximately 100 mg per day with dietary changes from 0 3 to 1 4 gm calcium) When Dr Connor and Dr Hopkins tried similar experiments (on ambulatory subjects) the urinary variations showed an increment of 100 to 150 mg of calcium when to a low calcium diet was added one quart of milk per day[34] In bed ridden patients with fractures who had considerable areas of their bodies immobilized in casts and hence *were excreting much larger than normal quantities of urinary calcium* (clearly from disuse rarefaction) we found no important change in calciuria resulting from dietary shifts of 200 mg to 2200 mg calcium—

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[31]c Orr W J Holt L E Jr Wilkins L and Boone F H The Relation of Calcium and Phosphorus in the Diet to the Absorption of These Elements from the Intestine *Am J Dis Child* 28 574 (1924)

d Farquharson F R Salter W T and Aub J C Studies of Calcium and Phosphorus Metabolism the Effect of Ingestion of Phosphates on the Excretion of Calcium *J Clin Investigation* 10 251 (1931)

[32] Gamble J L *Chemical Anatomy Physiology and Pathology of Extracellular Fluid A Lecture Syllabus* Boston Harvard Medical School Department of Pediatrics (Chart 36) (1939)

[33] Knapp E L Factors Influencing the Urinary Excretion of Calcium I In Normal Persons *J Clin Investigation* 26 182 (1947)

[34] Hopkins T R and Connor T B Unpublished data

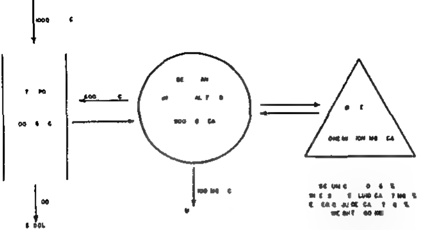

Fig 1 Schematic Diagram of the Approximate Quantitative Aspects of Calcium Homeostasis in Man

all in the form of milk When similar additions of calcium were made in the form of lactate however thus making a great preponderance of calcium over phosphorus such dietary change was reflected by an increase of 150 mg or more in the urinary calcium[34] We were surprised to note no change in the quantity of urinary calcium when alkali such as sodium bicarbonate or sodium citrate were administered to such patients during constant diet periods[5]

However there is quite definitive evidence that the uptake of ingested calcium does change under varying environmental circumstances In experimental vitamin D poisoning hypercalcemia vicarious calcification and renal damage are produced far more vigorously and rapidly if a liberal calcium intake is provided The child must absorb much more calcium from a daily quart of milk (or lose less of his secreted intestinal juice calcium) than does the normal adult because his calcium balance is obviously strongly positive McCance Widdowson and Lehmann found that elevation of the protein in the diet favored absorption of calcium as judged by increase

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Howard J E Parson W and Bigham R S Jr Studies on Patients Convalescent from Fracture III The Urinary Excretion of Calcium and Phosphorus *Bull Johns Hopkins Hosp* 77 291 (1945)

Harris L J and Innes J R M The Mode of Action of Vitamin D Studies on Hypervitaminosis D The Influence of the Calcium Phosphate Intake *Univ of Cambridge Institute of Animal Path Report* 2 151 (1931)

in the urinary and reduction in the stool calcium. Their interpretation of this was that the amino acids derived from the protein favored calcium transfer from the intestine. Contrariwise, Emerson and Beckman[8] found that calcium absorption in nephrotic children was practically nil when protein absorption was poor, but in an induced remission when stool nitrogen fell, the calcium absorption rose promptly to normal levels.

Can excessive absorption of calcium from the intestinal tract ever result in hypercalcemia? It seems reasonably certain that in vitamin D poisoning *provision of large quantities of calcium in the diet contributes heavily to* the hypercalcemia and hence is harmful. But whether excessive ingestion of calcium to the *normal* person can produce hypercalcemia seems doubtful. The instances of hypercalcemia reported by Burnett and associates[8a] (in which paper the author was a participant) and those described by Mulholland[9] were associated with prolonged ingestion of large quantities of milk and alkali taken for relief of peptic ulcer symptoms. In Mulholland's patients the pathological situation seemed partially, at least, reversible, renal function improving coincident with return of normocalcemia when the tremendous milk intake was stopped. Can one possibly pile in enough calcium so that Hendricks' system will be completely satiated and thus set the calciostat at a higher than normal level? This seems hard to visualize unless some associated renal lesion is present which restricts the urinary output of calcium.

One such patient[9] of the authors was explored, the parathyroids were found markedly enlarged and microscopically hyperplastic, and three glands were removed and three fourths of the fourth gland. Despite cessation of milk and alkali therapy no fall in serum calcium resulted from this procedure until some four months later when there was abrupt fall to tetanic levels requiring oral support by calcium salts. No reasonable explanation for this trend of events was apparent then, nor is it now. Perhaps there are some persons whose intestinal tract is so constituted as to absorb large quantities of calcium if such is provided in the diet, whereas most of us absorb but little calcium no matter how much is eaten unless the bones have a need for it. But it seems to us that one must also postulate a reduced capacity of the kidneys to respond to hypercalcemia by increased hyper

[8] Emerson, K., Jr., and Beckman, W. W.: Calcium Metabolism in Nephrosis. Description of Abnormality in Calcium Metabolism in Children with Nephrosis. *J. Clin. Investigation* 24:564 (1945).

[8a] Burnett, C. H., Commons, R. R., Albright, F., and Howard, J. F.: Hypercalcemia without Hypercalcuria or Hypophosphatemia, Calcinosis and Renal Insufficiency. *New England J. Med.* 240:787 (1949).

[9] Mulholland, H. B.: Hypercalcemia with Renal Insufficiency. *Trans. Am. Clin. and Clim. Assoc.* In press (1952).

calciuria Renal damage may be of a type resulting in retention of sodium but some cases are sodium losers and the same has been reported for potassium A renal lesion which results in poor calcium excretion to the usual stimuli such as acidosis or hypercalcemia might then quickly saturate the skeletal surfaces and a vicious circle be set up Some of the cases in Burnett s series may have had such a set of circumstances involving the first clinical picture One visualizes here more or less the opposite of the congenital renal situation in which excessive quantities of both calcium and phosphorus are excreted at normal levels of serum calcium and phosphorus —resulting in great drains on the skeletal minerals and eventually skeletal rarefaction and usually osteomalacia

To summarize then it may be said that in health the factors regulating the overall uptake of calcium by the gastrointestinal tract are but poorly understood but among other more local factors the status of the bones—i e how much they remove or yield during passage of the extracellular fluids past their immediate environment—probably does in some way affect the absorption of available elements in the upper gastrointestinal tract Diarrheas are of course notorious depletors of skeletal lime salts by virtue of reduced absorption due to increased peristaltic movements probably by increased intestinal secretions (the electrolytes of which are less resorbed) and in the cases of steatorrheas by the additional vitamin D deficiency So far as we are aware there is no evidence that active specific excretion of calcium ever occurs in the colon

THE KIDNEY AND CALCIUM HOMEOSTASIS

Let us turn now to the kidney and its relation to calcium homeostasis It has been mentioned previously that normal persons usually excrete about 100 mg of calcium in their urine The present concept is that glomerular fluid is a serum ultrafiltrate or diffusate and if 125 cc are created per minute then 7 5 litres of fluid are presented to the tubules per hour or 180 litres per day This would contain 9 grams of calcium or 12 5 grams depending upon the figure 5 or 7 mg of ultrafilterable of calcium per 100 cc of serum In any event more than 99 per cent of the calcium must normally pass back into the system through the urinary tubules and the amazing feature is the constancy of the minuscule quantity which appears in the urine One wonders whether the premises on which this concept of calcium is based are correct It becomes even more amazing when one notes that in the presence of hypercalcemia (and we mean by this increased ultrafilterable serum calcium) although hypercalciuria invariably results the quantitative increase in the urinary calcium excretion is so exceedingly small Even in severe hypercalcemias of 16 to 18 mg per 100 cc there is rarely more than 1 gram of calcium in a 24 hour urinary collection de

spite the fact that under these conditions *10 more grams* are supposedly presented to the tubules in the glomerular filtrate Conversely the smallest tendency toward acidosis[9] or the imposition of skeletal immobility [35,41] or thyrotoxicosis[4] (all of which are accompanied by rarefaction) will without detectable hypercalcemia increase the urinary calcium almost as much as will hypercalcemia itself These thoughts lead us to speculate whether there may not be a specific excretory mechanism which governs the quantity of calcium appearing in the urine and that all the glomerular filtrate calcium is resorbed or diffused back into the system

It has been said that the parathyroid hormone when given to normal persons produces an increase in urinary calcium hours or even days before hypercalcemia is manifest [43] It is difficult to be certain of such a statement because a very small rise in the serum calcium (and our methods allow accuracy only to 0 2 mg per 100 cc of serum) should result in a great increase in the total calcium filtered through the glomeruli Some data on a *hypoparathyroid* patient of ours certainly do not suggest any important *direct* action on calcium excretion by the kidney at least when the serum calcium is low (Figure 2) The patient had surgical hypoparathyroidism and on a constant diet was given 3 cc of parathyroid extract intramuscularly every six hours for 48 hours The serum calcium level did not change nor did the urinary calcium excretion Meanwhile the serum and the urinary phosphorus levels showed changes of great magnitude as will be discussed under phosphate homeostasis

Whether or not the height of serum phosphorus *per se* affects the quantity of calcium excreted by the kidney we do not know But during the experiments in which phosphate was administered intravenously to patients *it was noted that though the serum calcium level did not fall (at least* no more than could be accounted for by dilution as measured by the hematocrit and the hemoglobin) the urinary calcium excretion did fall during the 24 hour period of elevated serum phosphorus level [8b] This was true in the

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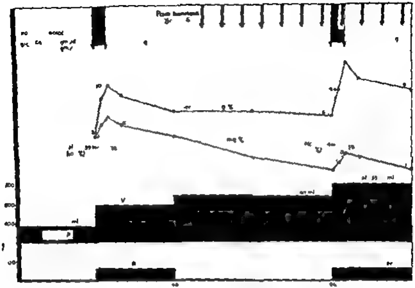
[9]Albright F and Reifenstein E C Jr *The Parathyroid Glands and Metabolic Bone Disease Selected Studies* Williams and Wilkins Co Baltimore p 251 (1948)

[41] Deitrick J E Whedon G D and Shorr E The Effects of Bed Rest and Immobilization upon Various Chemical and Physiological Functions of Normal Men Their Modification by the Use of the Oscillating Bed *TRANS MACY CONFERENCE ON METABOLIC ASPECTS OF CONVALESCENCE* 12 44-61 (1946)

[4] Aub J C Bauer W Heath C and Ropes M Studies of Calcium and Phosphorus Metabolism III The Effects of the Thyroid Hormone and Thyroid Disease *J Clin Investigation* 7 97 (1929)

[43]Albright F and Reifenstein E C Jr *The Parathyroid Glands and Metabolic Bone Disease* Williams and Wilkins Co Baltimore p 73 (1948)



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and also has been noted by Kyle[46] in a similar patient This rise in the urinary calcium excretion occurs despite a strong shift to a positive calcium balance and little or no noteworthy change in the serum calcium concentration [45 46]

Conversely we recently have noted in several patients with hypoparathyroidism who were being treated with vitamin D (100 000 to 300 000 units daily) *with or without* large doses of calcium salts orally that the urinary calcium excretion has been quite large (200 to 300 mg per day) in spite of a low serum calcium level in the range of 7 8 mg per 100 cc of serum (and with a low ultrafilterable calcium content from the serum) [31]

In any event the kidney appears to be *relatively* impotent or indifferent to gross elevations of calcium in the body fluids and to play but a minor role in the normal constancy of the serum calcium concentration One is reminded of a bathtub with a small overflow outlet If water continues to flow in from the spigots at any but the smallest rate the water will rise above the outlet and eventually overflow

The State of Phosphorus in Body Fluids

Phosphate is so much more widely dispersed than calcium in the cellular compartment (though most does reside with calcium in the skeleton)[21] and participates in such a multitude of metabolic reactions that its movements are far more difficult to define So far as we can tell the extracellular inorganic phosphorus is almost wholly ultrafilterable [2] even in pathological states—*except when there is coincident excessive hypercalcemia* [2] Acid soluble phosphorus other than inorganic phosphate has not been found in serum higher than 0 5 mg per 100 cc even in pathological states [47]

Factors Affecting the Phosphorus Balance

THE GASTROINTESTINAL TRACT AND PHOSPHORUS HOMEOSTASIS

In the normal person on an average diet approximately two thirds of the quantity of intake appears each day in the urine so that absorption must ordinarily be in keeping—i e two thirds of the ingested phosphorus When one precipitates the dietary phosphorus by feeding excesses of calcium iron or aluminum far less phosphorus appears in the urine But though diurnally the concentration of serum phosphorus fluctuates and is in general lower the fasting serum phosphorus level changes little if any [48] The situa

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[46] Kyle L K Personal communication

[47] Guest G M Personal communication

[48] Shorr E and Carter A C Aluminum Gels in the Management of Renal Phosphatic Calculi *J A M A* 144 1549 (1950)

tion is different in the patient with renal insufficiency where usually an abrupt fall of the elevated serum phosphorus level is obtained by the simple expedient of administering calcium, iron or aluminum salts (provided of course that much phosphorus is not being added to the system from the cells by a vigorous catabolic reaction). The kidney of the uremic patient seemingly cannot excrete much phosphorus, the gut continues to absorb it and the cellular compartment cannot or at least does not remove it.

In this connection it is of some interest that in obtaining control and corollary observations on the effects of intravenously administered calcium we administered by the same route a gram or more of phosphorus as neutral sodium phosphate over a 4 hour period to four persons. In the cases of the two normal subjects 100 per cent and 71 per cent of the injected amount appeared in the urine within 24 hours and the serum phosphorus level had returned to normal. In a mild diabetic regulated with diet alone and in a patient with hyperparathyroidism recovery of injected phosphorus was 68 per cent and 74 per cent respectively. When we have been compelled to give large doses of calcium orally to patients with sprue *and these were greatly undernourished and depleted persons* the serum phosphorus level sometimes has fallen to 1 mg. per 100 cc. or lower and for days or weeks so little phosphorus has appeared in the urine that it has been barely detectable. Thus in contrast to its reaction to hypercalcemia the normal kidney appears decidedly interested in the level of the serum phosphorus and takes strong measures to counteract hyperphosphatemia.

THE KIDNEY AND PHOSPHORUS HOMEOSTASIS

Harrison and Harrison [*] have found that in the rachitic puppy vitamin D increases the renal tubular absorption of phosphorus and that this mechanism is an important if not major cause of the rise in the serum phosphorus level in response to D administration. McCance likewise noted in his case of adult vitamin D deficiency (resistance to vitamin D) that much less phosphorus was excreted in the urine when the patient was receiving adequate doses of the vitamin despite the fact that the serum phosphorus was far higher. He felt that this clearly indicated a change in phosphate threshold of the kidney.[45]

But in the therapy of hypoparathyroidism with vitamin D (100 000 to 300 000 units per day) the effect on renal excretion of phosphorus appears to be just the opposite. Here there is often a sharp fall in the elevated

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[*] Harrison, H. E., and Harrison, H. C.: The Renal Excretion of Inorganic Phosphate in Relation to the Action of Vitamin D and Parathyroid Hormone. *J. Clin. Investigation*, 20: 47 (1941).

serum phosphorus level *coincident with greatly* increased phosphaturia.[5] And this increased phosphaturia occurs usually before there is an appreciable rise in the serum calcium concentration. (It may be that hypercalciuria, however induced, is associated with relative hyperphosphaturia under circumstances *where the parathyroid glands are kept out of the picture*—i.e., when their functional status cannot be much altered as in hypoparathyroidism or parathyroid adenomata.[19b])

THE PARATHYROID HORMONE AND PHOSPHORUS HOMEOSTASIS

The evidence appears to us overwhelming that the parathyroid hormone exerts control over renal phosphorus excretion. In the normal individual administration of a single dose of the hormone intravenously results in but a minor increase in phosphaturia,[51–53, 54] perhaps because a maximal response to parathyroid hormone already is being involved or because the patient's own parathyroid glands cease to excrete. However, a similar dose of parathyroid hormone given to the hypoparathyroid patient with normal kidneys results in an immediate huge phosphorus diuresis[51] (Table IV).

Another example of this effect may be demonstrated by an experiment in which 3 cc. of parathyroid hormone was injected intramuscularly every six hours to a patient with hypoparathyroidism[19f] (Figure 2). Over a period of 48 hours the serum phosphorus level fell from 4.9 to 1.5 mg. per 100 cc. coincident with an increased phosphaturia of approximately 800 mg.[55] If one considers that the absorption of phosphorus from the gut has not been altered by the parathyroid hormone (which seems reasonable), then the urinary phosphorus that is excreted *more than* accounts for the fall in the serum phosphorus level. For in this 57 kg. woman there should have been only 600 mg. of phosphorus in her entire extracellular compartment.

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[5] Albright, F., and Reifenstein, E. C., Jr.: *The Parathyroid Glands and Metabolic Bone Disease: Selected Studies*, Williams and Wilkins Co., Baltimore, p. 130 (1948).

[51] Ellsworth, R., and Howard, J. E.: Studies on the Physiology of the Parathyroid Glands. VII. Some Response of Normal Human Kidneys and Blood to Intravenous Parathyroid Extract. *Bull. Johns Hopkins Hosp.* 55: 296 (1934).

[52] Jahan, I., and Pitts, R. F.: Effect of Parathyroid on Renal Tubular Reabsorption of Phosphate and Calcium. *Am. J. Physiol.* 155: 42 (1948).

[53] Milne, M. D.: Observations on the Action of the Parathyroid Hormone. *Clin. Sci.* 10: 471 (1951).

[54] Handler, P., Cohn, D. V., and DeMaria, W. J. A.: Effect of Parathyroid Extract on the Renal Excretion of Phosphate. *Am. J. Physiol.* 165: 434 (1951).

[55] This calculation is based on the quantity of phosphorus excreted in the urine in the 24 hours before the first intravenous calcium test is given, since in our experience the serum calcium and phosphorus level and the *urinary excretion of these substances* return to baseline values 24 hours after the intravenous calcium test has been completed.

TABLE IV

The Effect of Parathyroid Extract on the Urinary Excretion of Phosphorus and on the Serum Levels of Phosphorus and Calcium of a Patient with Hypoparathyroidism

Urine			Serum		
Time	Volume	Phosphorus	Time	Phosphorus	Calcium
	(cc)	(mg/hr)		(mg/100 cc)	(mg/100 cc)
7-8 A.M.	475	1.8	—	—	—
8-9 A.M.	165	1.0	—	—	—
9-10 A.M.	100	1.2	—	—	—
10-11 A.M.	100	1.7	11:00 A.M.	5.6	6.9
Parathyroid Extract 40 Units (2 cc) Intravenously at 11:25 A.M.					
11-12 Noon	90	25.8	12:30 P.M.	5.1	6.7
12-1 P.M.	112	83.5	1:30 P.M.	5.3	6.9
1-2 P.M.	45	41.5	—	—	—
2-3 P.M.	40	25.9	—	—	—
3-4 P.M.	95	20.0	4:30 P.M.	5.2	6.7

(when the serum phosphorus level was 4.9 mg per 100 cc) and so some phosphorus must have been provided by the cellular compartment to prevent the serum phosphorus concentration from reaching a level of zero at the end of 48 hours. This is the same patient shown previously whose serum calcium level and urinary calcium excretion were unaffected by the intravenous parathyroid hormone. It would appear to us that parathyroid hormone exerts a profound effect on the renal excretion of phosphorus especially manifest in the hypoparathyroid individual.

For purposes of the discussion that we expect will follow it might be well to include here some observations on the serum and urinary phosphorus values in patients from whom a parathyroid tumor recently has been removed. It has been observed for a long time that in some of these patients the serum phosphorus level will remain low for months postoperatively when all other signs of active hyperparathyroidism have disappeared. Interestingly enough when we tested two patients within the first 10 days after operation with the intravenous calcium test all of the urinary phosphorus and the serum phosphorus responses were the same as before operation—i.e., an insignificant rise in the serum phosphorus level and a small fall or even a rise in the urinary phosphorus excretion—just as though the phosphorus metabolism were still under hyperparathyroid control though the serum calcium and the urinary calcium values had fallen to normal or subnormal levels. However when retested several weeks later all of the

responses were in the normal range whether or not the serum phosphorus level had remained low or had returned to normal.[19b]

The Homeostasis of Phosphorus in Body Fluids

But that the kidney alone sets the "phosphastat" for the serum (or is it self uninfluenced by other factors in so setting it) seems unlikely. The serum phosphorus level of the normal infant runs consistently higher than that of the child, which in turn is higher than that of the adult, and in old age the serum phosphorus concentration tends to fall still lower. Albright has suggested that the growth hormone has something to do with this, and indeed most patients with active eosinophilic adenomas of the anterior pituitary gland do seem to have higher serum phosphorus levels than other members of their corresponding age group.[56] One gets the impression that the cellular phosphate (and perhaps bone takes part, but I do not as yet see just how) participates in the setting of the serum phosphorus level. The serum phosphorus level tends to be low in depleted persons when they are in nitrogen equilibrium and thus not expending their cell substance for energy. In the patient recovering from diabetic acidosis under insulin therapy the serum phosphorus values fall to very low levels with a negligible urinary phosphorus excretion, as if the phosphorus were rapidly disappearing cellward.[57, 58] We get the impression that the cells behave toward serum phosphorus homeostasis in much the same fashion as they appear to do in regard to potassium—they will support the serum phosphorus level until their own stockpiles reach a certain minimum, but thereafter they stop supporting it. Perhaps this thesis readily can be refuted by observations of which I am unaware on rachitic children, for an example, if studies on total muscle phosphorus have been carried out.

In connection with the serum phosphorus level it may be of some interest to recall the *rise* in the serum phosphorus concentration which occurs *when the serum calcium level is elevated by the intravenous* administration of calcium salts. This was first noted by Salvesen in the dog[59] and later by

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[56] Reifenstein, E. C., Jr., Kinsell, L. W., and Albright, F.: Observations on the Use of the Serum Phosphorus Level as an Index of the Pituitary Growth Hormone Activity; the Effect of Estrogen Therapy in Acromegaly. *J. Clin. Endocrinol.* 6: 4 0 (1946)

[57] Danowski, T. S., Hald, P. M., and Peters, J. P.: Sodium, Potassium and Phosphates in the Cells and Serum of Blood in Diabetic Acidosis. *Am. J. Physiol.* 149: 667 (1947)

[58] Guest, G. M., and Rapoport, S.: Electrolytes of Blood Plasma and Cells in Diabetic Acidosis and During Recovery. *Proc. Am. Diabetes Assoc.* 7: 95 (1948)

[59] Salvesen, H. A., Hastings, A. B., and McIntosh, J. F.: The Effect of the Administration of Calcium Salts on the Inorganic Composition of the Blood. *J. Biol. Chem.* 60: 327 (1924)

Bassett s group [20] it has been seen invariably by us in all *normal* individuals [9b] The quantitative aspects and the time relationships of this rise in the serum phosphorus level make it impossible to account for the rise by the small fall in the urinary phosphorus excretion which accompanies it in the normal individual [19b, 8] The fall in urinary phosphorus excretion following hypercalcemia has been attributed to a cessation or a reduction of parathyroid function [19b, 2] and perhaps the rise in the serum phosphorus level also is caused by this but if so there must have been a reaction in some cellular compartment phase to provide the PO_4 to the extracellular compartment Furthermore it seems unlikely that an abrupt shutting off of the parathyroid hormone excretion accounts for the whole rise in the serum phosphorus level following hypercalcemia because a sharp rise in the serum phosphorus level has been seen also in some cases of severe hypoparathyroidism where presumably there is no parathyroid tissue to turn off

Summary of Calcium and Phosphorus Homeostasis

To summarize these remarks has proven very difficult and awkward It may be said that

1 Calcium is transported in the serum partly as proteinate Interstitial fluid calcium is normally somewhere between 5 5 and 7 mg per 100 cc of fluid as it leaves the capillaries Additions to the circulating calcium may come from the gut or the bones subtractions may go to the bones into the gut or into the urine Additions of considerable quantities of calcium can be carried by normal serum (unless the serum phosphorus is well above normal concentration) before any mechanical factors within the serum itself disclose changes in the ultrafilterable fractions of calcium or phosphorus

2 Normally the serum calcium concentration is a closely guarded physiological constant and factors operating toward this constancy include the gut the kidneys and the bones but by far the largest potential operator in this constancy is the skeleton Hendricks theory of a vast number of calcium molecules on the surfaces of the skeletal apatite offers an attractive visualization of how bones can function so rapidly to so large an extent in this mechanism The kidneys can and do take care of small additions to the system of calcium which would otherwise result in hypercalcemia but any large ingress cannot be overcome completely by the renal excretory mechanisms and hypercalcemia results We know of no pathological situation wherein excessive absorption of calcium occurs from the gut at least with absorption of such magnitude as to result in hypercalcemia

3 Therefore only alterations in the skeletal physiology can change the serum calcium level materially and when one finds abnormalities of the serum calcium (hyper or hypocalcemia) factors working at the skeletal level have provided the change Thus there are clinical abnormalities such as dietary inadequacy intestinal absorptive defects or disturbances acting through the kidneys which can drain the system of calcium so that the skeleton eventually suffers severe rarefaction and clinical hypocalcemia is reached Or hypocalcemia may result at the skeletal level from pathological function either primary or secondary such as in rickets or hypoparathyroidism But hypercalcemia (elevation of the interstitial fluid calcium) must so far as we are aware originate with an abnormality at the skeletal level in such conditions as hyperparathyroidism destructive skeletal cancer disuse atrophy and vitamin D poisoning We believe that the same mechanism must hold for the as yet unexplained hypercalcemias that are seen with sarcoidosis and some neoplasms of the lung

4 The transport and serum homeostasis of phosphorus are governed ultimately by the same type of factors as those that play a role for calcium but the kidney seems to have a considerably more prominent place in determining the level of the serum phosphorus than in regulating the level of the serum calcium However here too one gets the impression that phosphate metabolism at the cellular level ultimately sets the serum phosphorus level probably through some equilibrium between organic and inorganic intracellular phosphorus with the latter determining the extracellular phosphorus concentration Any changes in serum phosphorus concentration exerted by extraneous factors such as growth hormone parathyroid hormone age acidosis and so forth affect the cell metabolism as well as the renal mechanisms

It should be emphasized again that the writer is well aware of the inadequacies and flaws of this review In it however there should be enough material to provoke discussion and clarification by others better qualified which together with the data on diseases involving pathological calcium and phosphorus metabolism should indicate where our present knowledge is inadequate and in what directions our efforts should be extended to achieve a better understanding of the problems

Conference Discussion

Armstrong Thank you Dr Howard I think it would be appropriate now to ask for some general comments and discussion about points

One reason for the difficulty in accepting the simple chemical equilibrium theory is that it is extraordinarily difficult *in vitro* to bring a solution of tricalcium phosphate into equilibrium with the solid phase This has caused difficulty to a great many investigators and still causes difficulty so that it seems hard to accept the idea that the attainment of equilibrium occurs rapidly in the living organism that it is possible for the blood *calcium to be readjusted from moment to moment by the simple process* of halisteresis

Another reason for not believing in halisteresis is that if the calcium X phosphate ion product is lowered over a long period of time as is the case in experimental rickets one would expect that all of the remaining bone salt would be dissolved out of the bones in an effort to keep up the ion product in the fluids of the body Actually we all know that this does not occur We know that if a rat is made rachitic by putting it on a *diet high in calcium and low in phosphorus the ion product is greatly* reduced to the extent that the fluids from the animal will no longer calcify rachitic cartilage *in vitro* But we also know that the process does not result in the solution of bone salt that is left in the bone at the time the animal's diet is changed In other words putting an animal on a rachitogenic diet stops new calcification but it does not reverse the process and lead to decalcification or to the process that used to be known as halisteresis I prefer therefore to think—and I hope there will be some further discussion on this point—of the equilibrium between the solid phase of the bone salt and the liquid phase in the fluids of the body being mediated by some biological process beyond the simple solubility process that occurs in the test tube when an inorganic salt is brought into equilibrium with the liquid phase

Of course this is not new and many people have the concept of the equilibrium being mediated constantly by the parathyroid hormone This runs into some difficulty in parathyroidectomized animals because there is still an exchange of calcium between the body fluids and the bones even though the animal is completely free from parathyroid tissue *It is* therefore not sufficient to regard the parathyroid hormone as essential to an exchange of mineral between the solid and the liquid phase I am hoping that Dr Kramer will continue this part of the discussion *He has been in this particular field longer than I have and I think* we should have some clarification as to just what we are thinking about when we consider the equilibrium between the mineral in the bone and that in the fluids of the body

Armstrong Thank you Dr Kramer would you like to make a statement now ?

Kramer Yes I have been very much impressed by the work of Hastings and Huggins[60] on the replacement of artificially produced calcium deficiency in the serum presumably by solution of calcium from bone When I first read these observations I thought of the possible effect of parathyroidectomy and that it might give a clue as to the mechanism of this mobilization of calcium which brings about the rapid reestablishment of the normal plasma calcium level While the reports of Hastings and Huggins are very meager as to detail they do indicate that in the absence of the parathyroid glands normal calcium levels frequently are not attained and tetany develops in these animals

While it is true that *in vitro* it is possible to get the serum or plasma to retain more calcium or more phosphorus and maintain a higher Ca × P product with a certain amount of stability the fact remains that in the studies of Hastings and Huggins the level rose to a certain point to a normal Ca × P product and then did not go beyond it and that point varied depending upon whether the parathyroids were in or out so that there seems to be some mechanism which determines the maximum level to which the product can rise It is probably not entirely humoral and is in part dependent upon intact parathyroid glands as well as upon adequate Vitamin D intake When we developed methods for determining citric acid we thought that the determination of serum citric acid might throw some light on this problem by showing that the plasma calcium could rise because of an increase in citric acid and therefore an increase in soluble un-ionized calcium The hypercalcemia of normal rabbit plasma is in part due to an increased serum citric acid level This however is not the case in the dog or in the child with hypercalcemia I was wondering whether in the dogs that had been used for this experiment there was any evidence histologically of removal of mineral without simultaneous removal of the organic matrix Dr McLean probably can answer this question

McLean In our experience none

Kramer I have a few points here and there that I jotted down as Dr Howard spoke In studying cerebrospinal fluid[61] as a possible example of a physiological ultrafiltrate of calcium and phosphorus and of a Donnan membrane equilibrium we could never account for the tiny amount of inorganic phosphorus found in the cerebrospinal fluid as compared for example to the amount of phosphorus that is found in the ultra

Hastings A B and Huggins C B Experimental Hypocalcemia *Proc Soc Exper Biol and Med* [illegible] 458 (1933)

[61] Pincus J B and Kramer B A Comparative Study of the Concentration of Various Anions and Cations in Cerebrospinal Fluid and Serum *J Biol Chem* 57 463 (1923)

filtrate of normal plasma. I was wondering if Dr. Howard would have something to say about that. Furthermore, in doing ultrafiltration experiments, we found a striking change in the pH of the ultrafiltrate; this led to the precipitation as calcium phosphate of part of the calcium which failed to precipitate with oxalate, and therefore we obtained low values for calcium in the ultrafiltrate. When we realized what was happening, adjustment of the ultrafiltrate pH gave us more nearly normal results.

Follis: May I just ask a question of Dr. McLean? Dr. McLean, do you then disagree with the postulate that there need not necessarily be bone destruction? Is it your point that there *must* be bone destruction in order to account for calcium and phosphorus being made available? Or do you contend, as I think Dr. Howard believes, that there need not necessarily be bone destruction, but that calcium and phosphorus may be liberated from bone, i.e., the bone crystal? In other words, one can regard it as a sort of modified halisteresis in the sense that adsorbed ions come off the crystal, which itself as well as the organic matrix need not be destroyed.

McLean: Our impression is that there must be an area of destruction of bone, including matrix, perhaps very sharply localized, in order to liberate the bone salt. This really goes back to the concepts introduced by Koelliker eighty years ago. He concluded that the osteoclast erodes bone by chemical means without specifying further the nature of the chemical action required. Later, others added the supposition that the action is a combination of that of an acid with that of a proteolytic enzyme. The acid dissolves the bone salt and the proteolytic ferment destroys the matrix. This is a crude idea as to how this kind of thing may occur. It is still our impression that something of the sort happens; that the bone tissue must be torn down, actually torn down, in order to put bone salt into solution as rapidly as it does get into solution if something happens to modify the level in the blood, as in the experiments of Hastings and Huggins.[0] I simply cannot picture replacement of serum calcium as rapidly as it was demonstrated by Hasting and Huggins on the basis of re-solution, simple solution from the solid phase of bone.

Neuman: I want to say that I have one area of complete agreement with Dr. McLean. I have also one area of disagreement: this matter of simple re-solution. It is not simple. We must accept the fact that a few litres of fluid flow over two acres of mineral surfaces—

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[6] Koelliker, A.: *The Normal Resorption of Bone Tissue and Its Importance in the Formation of Typical Bone Forms.* F. C. W. Vogel, Leipzig (1873).

Now, if the crystal does not exist as an entity, then neither does such a thing as a solubility product, and therefore I am not bothered now as I was a few years ago when the clinicians report high calcium and high phosphorus or low calcium and low phosphorus. I am convinced that the crystals present in the available skeleton adjust in composition to changing blood levels. It is true that it takes years to get final equilibrium in vitro because the crystals which are formed immediately readjust to the solution ad infinitum. They change in size; they even change in character. The blood-bone equilibrium, however, is dynamic, I believe.

During intermission, Dr. Robinson and I were scribbling on the board a modified Howard bathtub, in which the body fluids, the intestine and the kidneys, complicated by other active processes, regulate the level of calcium in the blood. Connected to the blood by a complicated physicochemical process is the bathtub, the skeletal reserve. If the calcium level in the blood is low, it, of course, drains the bathtub.

A further complication in this situation is the effect of old age. It is as if we possessed a Freon generator, a cooling system which gradually through life freezes the bathtub's contents so that, as the patient in this case becomes older, less and less of the bone is available for the regulation of the body fluid level.

Finally, here is our point of agreement: the unfreezing of buried areas is a cellular process; in other words, to draw significantly from the calcium of the bone, you have to invoke cellular processes to make the rest of the bone available. I would not deny the importance of the cell in this regulation, but the immediate response that one gets when withdrawing calcium from the blood, as in the classical Hastings experiment,[17] as far as I am concerned, is not mediated by cellular

Fremont-Smith: You mean there is no nec... it?

Neuman: There is no need to invoke a ... a

Fremont-Smith: But your acres would ... cells to operate, would they not?

Neuman: That's right. The available cry... in themselves, but the total contribution is ... the blood level without destroying any ... crystals which are available to the fluids ... solution process is horribly complicated. It ... cium level, the phosphate level, and the car... sodium, the magnesium, and the other ion... the calcium to sodium ratio of blood decre...

bone and calcium will come off This is not a matter of dissolving an entity a we ordinarily do with table salt but a very complicated exchange equilibrium in a very complicated solid in a very complicated solution But because it is complicated and we cannot define it we should not throw it out That is my plea

Shorr Then you have various families of crystals which are more or less accessible

Neuman Actually I think the bone crystals are pretty much alike in all animal skeletons I think they are alike because the kidney the intestine and all the other homeostatic mechanisms maintain such a good balance that the body fluids are essentially constant in composition throughout life The crystal form is a reflection of that composition and if we do observe variability in the bone it is because we do indeed observe variability in the composition of the blood

Shorr But the crystals are variously accessible to the body fluids is that right?

Neuman Yes indeed In the center of a compact shaft as in the beautiful pictures of Amprino [3] and—

Robinson Engstrom [4] Zetterstrom [7] and LaCroix [8]

Neuman The European group yes They have shown by microradiography that some of the newer Haversian system are relatively translucent to x rays Well those are available to isotopes in the circulation Other Haversian systems appear opaque and have little water in them These appear inert unreactive unavailable

Harrison You would agree Dr Neuman would you not that in the infant you have probably the maximum surface of available calcium?

Neuman Yes

Harrison But it is particularly in the infant in whom the serum levels of calcium and phosphorus are so variable that variations apparently are not compatible with a simple equilibrium between the fluid and the bone

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[3] Amprino R Reconstruction and Distribution of Bone Mineral II With Radioautographic Technique *Z Zellforsch u mikroskop Anat Abt A* 37 [illegible]40 (195[illegible])

[4] Engstrom A Engfeldt B and Zetterstrom R Relation Between Collagen and Mineral Salts in Bone Tissue *Experientia* 8 [illegible] (195[illegible])

[7] Zetterstrom R Renewal of Phosphate in Bone Minerals I Renewal Rate of Phosphate in Relation to the Solubility of the Bone Minerals *Biochim et Biophysica Acta* 8 283 [illegible]93 (195[illegible])

[8] LaCroix P Autoradiographs of the Spongy Osseous Tissue *Experientia* 8 426-4[illegible]8 (195[illegible])

salt in tl e skelton The ı r d ct of Ca × P n tl e ser n n tl e nfa t na ary from as lo v as 10 to as h gl as 80 I am ref rr ng to tl e con nt a t ons of calc m and norgan ı hosı horus n the serum U ler certa n c rcum stan es n nfa cy tl e level of the serum calc ma l e a low as 4 5 mg per 100 v l a serum ı l o pl oru concentrat on of ? to ? 5 n g ı cr 100

Foll s Yo l a e ı d fferent s tuat on ther of course You l a e a c n tantl ncrea a unt of rgan c n atrix h l ı a lal le fo deı o t on of n neral n the nfa t v l cl vou d ot l a e n the ad l

Harr so I tl k a a t all oe l ack to Dr McLean s dea tl at tl e orga c pro e e n tl e l one are ı rol al l much n ore mı orta t tl e ma tenanc f tl e er m cal m and ı ho ı hor s levels tl ı tl e n organ c ı roce ses

Ac Ma I g ba k tl u l You are st ll in ol ng a solu l t property Yo are a ng tl at f tl e alue are lo ver tl ere ca l e no l one or tl ere l oul l not l e l one and tl e only t me the alues c n l e lo er when the l one o e Th t s not tr e We kno tl at f l one s ı laced n water o d ot get the al es of l lood b t ou t so e th ng al o t ı te tl l oth n calc um and n pl osphorus of tl e l lood val An l k o also th t you can n x oncentrat on of calc un and ı l ı h ru that are ucl greater than fi l n ı la n a and tl ey do not ı rec p tate ther

Harr so Tha I am ure of hat the other part

A a If o thro o t tl e lea of a l m ted sol l l t tl ere no r ı on tl e world wl th r l o ld not l e bo e at ı le el of 2 of cal u per 100 of e um

Harr so We l ı be n terested part cularl ı group of nfan w tl d arrl eal d ease ho ha e l een g en ex e e amount of sod un salt Tl e e fants sl ow h pokalem a l pocalcem a and h poı l o ı l a ten a Tl ere s o e le ce of rap d restorat o of extra cell lar ca n and pho ı l or fr n the skeleto Tl e le el seem to be stat c

Ne a Ho v lo doe t take to reacl tl e stat c tuat on

Ha r so Tl e le el ren lo for se eral da and of cour e we l a to nterr ı t tl tate w h theraı Wh does ot tl e sk l on p ı ort the serum le el of calc u a l ı l o ı hor s more adequatel ? We l ı e seen lar tates o ca onall lur tl e co rse of e ere n fect o s

Ac a What l ı ı e to tl e excret o of calc um in ucl ca e

Harr so Tl e ur ır excret o of cal pl o pl oru e tren el lo

THE EFFECT OF VITAMIN D ON THE SOLUBILITY OF CALCIUM AND PHOSPHORUS IN SERUM

ALBERT E SOBEL

From the Department of Biochemistry The Jewish Hospital of Brooklyn Brooklyn New York

Armstrong Dr Sobel do you wish to make some remarks?

Sobel I would like to add something to the subject When one is discussing the serum calcium concentration it is worth while to consider the subject in the bird Under the influence of estrone one can get serum calcium level as high as 600 mg per 100 cc in the duck and during the period of egg laying the serum calcium level rises tremendously in most birds The explanation is a physicochemical one namely that in the blood a compound is present which permits the formation of an un ionized complex with calcium This substance is supposed to be a protein

Shorr Vitellin

Sobel Yes What I wish to point out is that there must be factors which increase the total solubility of the Ca × P product that are influenced by vitamin D First I shall show a table illustrating the relationship between the dietary calcium phosphate and vitamin D and the serum calcium and inorganic phosphate level in the growing rat (Table V) [a] We see that in the absence of vitamin D we get certain serum calcium and phosphate levels which are related to the dietary calcium and phosphorus that is the less the calcium in the diet compared to the amount of phosphate the less calcium in the serum and the higher the serum phosphate compared to the calcium In the absence of vitamin D the product of the serum Ca × P is relatively low All this time there is a negative balance The bones are giving off calcium and phosphorus yet maximal blood levels such as might be attained when vitamin D is given are not reached With vitamin D both the serum calcium and the inorganic phosphate levels are higher and particularly that member of the pair goes up which happens to be low All this time there is a positive balance The bones are being enriched with calcium and phosphate

Now let us look at rickets produced on a diet very low in phosphate

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Sobel A E and Hanok A Calcification of Teeth I Composition in Relation to Blood and Diet *J Biol Chem* 176 1103 (1948)

salt in the skelton The product of Ca × P in the serum in the infant may vary from as low as 10 to as high as 80 I am referring to the concentrations of calcium and inorganic phosphorus in the serum Under certain circumstances in infancy the level of the serum calcium may be as low as 4 5 mg per 100 cc with a serum phosphorus concentration of 2 to 2 5 mg per 100 cc

Follis You have a different situation there of course You have a constantly increasing amount of organic matrix which is available for deposition of minerals in the infant which you do not have in the adult

Harrison I think again it all goes back to Dr McLean's idea that the organic processes in the bone are probably much more important in the maintenance of the serum calcium and phosphorus levels than the inorganic processes

Neuman May I go back though? You are still involving a solubility property You are saying that if the values are lower there can be no bone or there should not be bone and the only time the values can be lower is when the bone is gone That is not true We know that if bone is placed in water you do not get the values of blood but you get something about a tenth both in calcium and in phosphorus of the blood values And you know also that you can mix concentrations of calcium and phosphorus that are much greater than we find in plasma and they do not precipitate either

Harrison That I am sure of but the other part—

Neuman If you throw out the idea of a limited solubility there is no reason in the world why there should not be bone at a level of 2 mg of calcium per 100 cc of serum

Harrison We have been interested particularly in a group of infants with diarrheal diseases who have been given excessive amounts of sodium salts These infants show hypokalemia hypocalcemia and hypophosphatemia There is no evidence of rapid restoration of extracellular calcium and phosphorus from the skeleton The levels seem to be static

Neuman How long does it take to reach the static situation?

Harrison The levels remain low for several days and of course we have to interrupt this state with therapy Why does not the skelton support the serum levels of calcium and phosphorus more adequately? We have seen similar states occasionally during the course of severe infections

Neuman What happens to the excretion of calcium in such cases?

Harrison The urinary excretion of calcium and phosphorus is extremely low

THE EFFECT OF VITAMIN D ON THE SOLUBILITY OF CALCIUM AND PHOSPHORUS IN SERUM

ALBERT E. SOBEL

From the Department of Biochemistry, The Jewish Hospital of Brooklyn, Brooklyn, New York

Armstrong: Dr. Sobel, do you wish to make some remarks?

Sobel: I would like to add something to the subject. When one is discussing the serum calcium concentration it is worth while to consider the subject in the bird. Under the influence of estrone one can get serum calcium levels as high as 600 mg. per 100 cc. in the duck, and during the period of egg laying the serum calcium level rises tremendously in most birds. The explanation is a physicochemical one, namely that in the blood a compound is present which permits the formation of an un-ionized complex with calcium. This substance is supposed to be a protein.

Shorr: Vitellin.

Sobel: Yes. What I wish to point out is that there must be factors which increase the total solubility of the Ca × P product that are influenced by vitamin D. First I shall show a table illustrating the relationship between the dietary calcium phosphate and vitamin D and the serum calcium and inorganic phosphate level in the growing rat (Table V).[1] We see that in the absence of vitamin D we get certain serum calcium and phosphate levels which are related to the dietary calcium and phosphorus; that is, the less the calcium in the diet compared to the amount of phosphate the less calcium in the serum and the higher the serum phosphate compared to the calcium. In the absence of vitamin D the product of the serum Ca × P is relatively low. All the time there is a negative balance. The bones are giving off calcium and phosphorus. Yet minimal blood levels such as might be attained when vitamin D is given are not reached. With vitamin D both the serum calcium and the inorganic phosphate levels are higher and particularly that member of the pair goes up which happens to be low. All the time there is a positive balance. The bones are being enriched with calcium and phosphate.

Now let us look at rickets produced on a diet very low in phosphate

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[1] Sobel, A. E., and Hanok, A.: Calcification. [illegible] Composition [illegible] Relation to Blood and Diet. J. Biol. Chem. 176: 1103 (1948).

TABLE V

The Effect of Vitamin D on the Relationship of the Serum Calcium and Inorganic Phosphorus Levels to the Calcium and Phosphorus Composition of the Diet in Rats

Diet		Serum*			
		Without Vitamin D		With Vitamin D†	
Calcium	Phosphorus	Calcium	Phosphorus	Calcium	Phosphorus
(%)	(%)	(mg/100 cc)	(mg/100 cc)	(mg/100 cc)	(mg/100 cc)
1 20	0 121	11 7	2 1	13 3	3 4
0 20	0 124	9 4	4 7	11 1	6 0
0 03	0 759	5 6	7 5	8 8	8 4

Wistar rats 23 days old were placed on the diet for 30 days Each of 6 litters was split among the 6 groups
*Mean values for serum
†100 I U of Vitamin D daily
[From Sobel A E and Hanok A Calcification of Teeth I Composition in Relation to Blood and Diet *J Biol Chem* 179 205 (1949)]

although adequate in calcium [9] where in the absence of vitamin D rickets develops but with a certain amount of new calcification When such animals are given vitamin D the Ca × P product of the blood is increased and the rachitic condition heals but the total amount of calcium and phosphate in the bones actually is decreased You recall these experiments Dr Harrison?

Harrison Yes The bone is redissolved apparently

Sobel Thus there seem to be factors that maintain the solubility of calcium and the phosphate in the blood We would like to think of these factors in terms of compounds that result in un ionized calcium and un ionized phosphate complexes Such compounds would permit a higher amount of calcium to be present without exceeding what we consider to be the solubility product principle One compound has received particular attention that is citrate and another series of compounds namely proteins also have received some consideration We really know very little about the dissociation constants of the various calcium protein complexes that are present in the blood All that we do know is the dissociation constant of

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[9]Coleman R D Becks H Kohl F V and Copp D H Skeletal Changes in Severe Phosphorus Deficiency of the Rat Tibia Metacarpal Bone Costochondral Junction Caudal Vertebra *Arch Path* 50 209 (1950)

the average of the calcium protein complexes that are present in the average normal blood from the studies of McLean and Hastings.[1,2]

It is likely that the compounds which permit higher solubility, let us say under the influence of vitamin D, are the very compounds that mediate the solution of calcium phosphate either at the expense of the bone or at the expense of the diet. Vitamin D increased the product at the expense of the bone in Dr. Harrison's study. In our experiments (Table V) it increased the product at the expense of the diet. The common denominator with normal as well as with high vitamin D dosage is the increase in the blood $Ca \times P$ product. Therefore the common factor is probably a compound that is present in the blood which permits higher solubility to exist.

This explanation brings out another aspect of the problem of serum levels and indicates that the mechanism is not as simple as increased resorption in the kidney or increased excretion or decreased secretion. Certainly one cannot explain the changes in the serum calcium level in the bird, which are of very marked degree, by such mechanisms. I cannot draw any conclusion except that the amount of calcium and phosphate in the blood must be regulated in some manner by compounds that are present in the blood, whose appearance can be stimulated by hormones in the case of bird, and by vitamin D and possibly other factors in the case of humans.

Conference Discussion

Kramer: I am more and more impressed with the importance of the biological factors in restoring calcification. In a case of osteopetrosis complicated by severe rickets we were able to produce very definite x-ray evidence of very extensive healing without any appreciable rise in the calcium or inorganic phosphorus concentration in the blood or change in the serum protein or albumin globulin ratio.

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[1] McLean, F. C., and Hastings, A. B.: The State of Calcium in the Fluids of the Body. I. The Conditions Affecting the Ionization of Calcium. J. Biol. Chem. 108, 285 (1935).

[2] McLean, F. C., and Hastings, A. B.: A Biological Method for the Estimation of Calcium Ion Concentration. J. Biol. Chem. 107, 337 (1934).

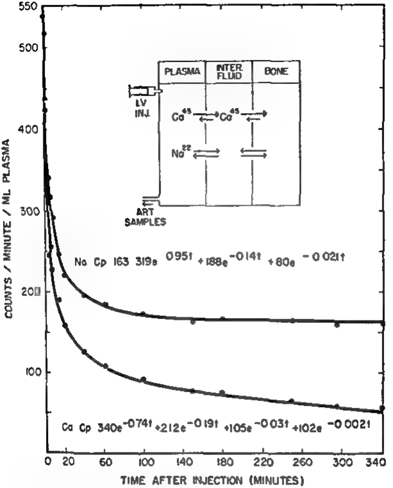

Fig 3 Arterial Plasma Concentration Curves of Radiosodium and Radiocalcium Injected Simultaneously into Two Dogs

The diagram illustrates the principle of the experiment and the arrows in the diagram indicate the relative magnitude and direction of movement of the labeled ions between the compartments of the body

agrees very well with previous data on sodium transcapillary migration rates In the two animals in which the sodium and calcium transcapillary migration rates could be obtained simultaneously a very close agreement

TABLE VI

The Fractional Turnover Per Minute of Plasma Calcium and Sodium in Dogs Given Radiocalcium Alone or With Radiosodium

Isotope	Number of Dogs	Turnover Rate (%)
6 Animals		
Calcium		51 9
Animal 1		
Calcium		60 1
Sodium		56 4
Animal 2		
Calcium		30 3
Sodium		29 7

was found between the rates. There may be some reason to question the validity of transcapillary migration rates calculated from arterial plasma concentrations If the results are questioned on that score we must recognize that these are minimum rates of turnover But this is not a point of particular importance to our present discussion

I have used the same data to calculate the quantities of calcium and sodium turned over per minute by the skeleton (Figure 4) I have had to assume here that the calcium and sodium not present in the extracellular fluid are present in bone In order to make these calculations we used a variant of the isotope dilution equation shown in Figure 4 What actually is done is to calculate the quantity of calcium or sodium through which the injected radioisotope is distributed in order to give the measured activity per milligram of plasma calcium or sodium at the indicated times and then to substract from this total quantity the amount of sodium or calcium that is present in the extracellular fluid the remainder that is obtained is the amount which is derived from the skeleton The application of this equation requires the assumption that the specific activities of the plasma and of the interstitial fluid are equal at any given time This assumption certainly is not true over the very early minutes of the experiment but the error introduced rapidly lessens with time In the case of sodium by 150 minutes we have an equilibrium concentration hence there is no error Because of the rapidity of the adjustment between the plasma and the inter

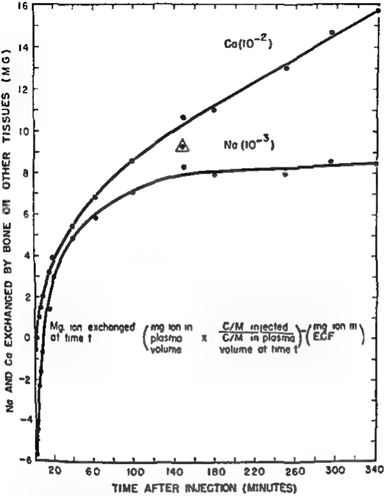

Fig 4 The Turnover of Skeletal Calcium and Sodium in Dogs

The dogs weighed approximately 20 kilogram The point in the triangle gives the mean value for calcium obtained from studies in 6 animal

titial fluid I believe there is only a small error in the calcium data beyond 100 minutes You can see at 150 minutes about 0 9 gram of calcium had been exchanged but at this time nearly 9 grams of sodium had been exchanged The mineral phase of the skeleton is about 3 per cent calcium and about 0 7 per cent sodium Here we have an example of a very much larger fraction of the skeleton sodium than of the skeleton calcium being

turned over This result is I believe consistant with the general thesis of Hendricks as to the surface location of sodium in the bone salt thus making it more readily available for exchange

As I say the real point of showing these data is to indicate that there are rapid processes by which calcium does move between plasma and interstitial fluid With the two acres or the eighty acres or whatever the area of the bone mineral surface is processes of solution and precipitation as well as of exchange certainly give an opportunity for rapid adjustments in the distribution of the mineral parts of the skeleton between the skeleton and the body fluids I am now going to call on Dr Rubin because I think he can carry this topic a great deal further

Conference Discussion

McCance May I interpose a question or a remark here? It seems to me that we are discussing two quite different biological phenomena First of all there is the proceeding which was described by Hastings in which if you remove calcium from the circulating fluid the deficiency is rapidly made good The second is the maintenance of what I might call steady states by biological processes at various levels of serum calcium and phosphorus Now until we get quite clear that we are thinking of two separate things I do not think we are going to make much progress biologically

Armstrong I would suppose that those processes are actually to a high degree related

McCance Well they may be related but they do seem to me to be two rather distinct processes Something is fixing the steady state

Armstrong It is quite true that we are dealing with an adjustment to a steady state The work earlier referred to was a disturbance of the equilibrium — if I am allowed to use equilibrium?

Neuman I am delighted I consider it an equilibrium condition

Reifenstein It is a disturbance of the steady state

McCance Presumably if you disturb the equilibrium of the steady state adjustments will be made to restore it i e to maintain the serum calcium at its old level whatever that may have been

Armstrong Are you talking about the bleeding experiments now?

McCance Yes

Shorr And isn t there a third consideration? If the experiments *in vitro* that Dr Howard reported are valid then the steady state is maintained at

a considerable degree of unsaturation under most conditions with which we are acquainted

Neuman I object! The problem of saturation of solubility is still hanging around and I wish we could get agreement on it It seems to me that what we have done here—is to hide ignorance with a term I am not much of a physical chemist but I do know that the principles of physical chemistry are very rarely applicable and I think this is one of the examples The solubility *principle* is one of the accepted things that I would not want to argue about but I would argue with its *application* in this case There are very rigorously defined conditions under which the solubility principle can be applied and they do not hold here

Armstrong I think we are all agreed that we cannot use solubility product in the classical physicochemical sense because we do not have a single solid phase and we have never really reached equilibrium both of which are required by definition for solubility product As you all recall some of us have tried to get around that difficulty by using ion product to describe whatever it is that we are talking about

Neuman But the application of the solubility principle doesn't hold It doesn't hold at all!

Shorr I merely refer to the fact that more can be held in solution in vivo regardless of what are the factors responsible

Neuman All right that is a demonstrable fact

Shorr I should think then that there are conditions in vivo where this state would also prevail

Neuman I agree

Shorr For example after removal of the kidneys the degree to which blood calcium and phosphorus levels may rise (without the calcium and phosphorus actually being precipitated as far as we can tell) indicates that whatever the factors there is a greater capacity on the part of the serum to hold calcium and phosphorus in solution than would be indicated by the values which ordinarily exist Can we say that these facts can be used as the basis for a discussion of the possible mechanism involved

Neuman Yes I do not want to be foolish about this but I also wish with apologies to the next speaker to make my point clear to this extent I am not endeavoring to belittle the importance of the cell but on the other hand because we do not understand some of the so-called simple chemical relationships here I do not think we should thereby bury our ignorance by calling it a biological process I want to know which is which we shall put

have to accept the fact that we do not know? We cannot describe the cellular effects because we do not know what is due to physical chemical events That is the problem to which I have been devoting my energies to try to learn what the cell is *not* doing so that what is left may be defined

Fremont Smith Ignorance does not have to be limited to the biological sphere [Laughter]

Armstrong I will say "amen" to your comment We have to make a diagnosis by exclusion here

DYNAMICS OF CALCIUM METABOLISM

MARTIN RUBIN[1a] RICHARD D THOMAS
THEODORE A LITOVITZ[1b] and CHARLES F GESCHICKTER

From the Chemo Medical Research Institute and the Department of Pathology Georgetown University Medical School and the Department of Physics Catholic University of America Washington District of Columbia

Armstrong Dr Rubin you may talk if you wish on either or both of the subjects upon which you prepared

Rubin Well this is a pleasant opportunity to display our innocence and our ignorance We have attempted to answer a number of questions concerning the dynamics of calcium metabolism mostly because we have been up to now completely unaware of the work that has been done elsewhere We haven t yet caught up with our reading

The studies which we have carried out of potential interest to this Conference Group are divisible into three segments The present discussion surveys some aspects of the dynamic metabolism of calcium as indicated by studies with radioactive calcium[45] In succeeding papers it may be possible to review some applications of synthetic chelating agents from a general point of view and more specifically as applied to calcium and magnesium metabolism

Biological Systems and Exponential Functions

Many natural phenomena and especially living biological systems frequently change as exponential functions The generalized expression for such change is given by the equation $C(t) = Ae^{kt}$

[1] Supported in part by grants-in-aid from the U S Atomic Energy Commission [Grant AT (30 1) 838 1[22] The National Cancer Institute the National Institute of Health Public Health Service and the Geschickter Fund for Medical Research Inc

[a] Associate Professor in Chemistry Chemo Medical Institute Georgetown University Medical School

[aa] Research Assistant Department of Pathology Georgetown University Medical School

[b] Instructor Department of Physics Catholic U [illegible] f America

[c] Professor of Pathology Georgetown Univer [illegible] ool

The changing function may be for example concentration C varying with time (t) In the present studies C is used to denote the concentration of radioactive calcium[45] The right hand side of the equation indicates that some quantity A of the changing material is being altered at a rate indicated by the magnitude of the rate constant b as a function of time (t) It may be noted that while the A term denotes a factor of quantity (or as it has been termed a compartment factor) and may vary with many external circumstances the b term represents a rate of change constant and is usually typical for a given physicochemical event or reaction

It should also be noted that a given biological system may show an overall pattern of change which is the sum of a number of individual reactions each having the generalized form presented in the above equation If this is the case (and it is usually the case in living systems) then identification and separation of the individual components of an overall reaction will depend on how different the rates of the individual reactions may be as well as on how fine a technique of measurement has been developed While the successful separation of an overall system may thus show a minimum number of individual reactions it by no means precludes the possibility that several reactions of the same rate are occurring simultaneously and indistinguishably With these considerations in mind we have attempted to study and identify the individual components of calcium metabolism using tracer methodology The details of this study have been recently presented[78]

Analysis of the Fate of Radiocalcium in the Blood of Adult and Young Animals

The function of the blood as the common carrier of metabolic activity indicated that study of the fate of calcium in the blood might be of significance In Figure 5 is illustrated the decrease in the concentration of intravenously administered tracer quantities of Ca^{45} as a function of time in groups of adult and young rabbits The rapidity of the turnover of individual blood calcium atoms is indicated by the fact that about 70 per cent of the injected dose of radiocalcium has disappeared from the circulatory system within five minutes It is also clear that the adult animals retained the administered dose of tracer material in the blood somewhat longer than did the young animals The significance of the difference may be apparent subsequently from our discussion

Mathematical analysis of these overall curves [*, 0] indicates that in reality

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[78]Thomas R O Litovitz T A Rubin, M I and Geschickter C F *Dynamics of Calcium Metabolism Time Distribution of Intravenously Administered Radiocalcium* *Am J Physiol* 169 568 575 (1952)

[79]Flexner L B Cowie D B and Vosburgh G J Studies on Capillary Permeability with Tracer Substances *Cold Spring Harbor Symp Quant Biol* 13 88 (1948)

[*] Siri W S *Isotopic Tracers and Nuclear Radiations with Applications to Biology and Medicine* McGraw Hill New York (1949)

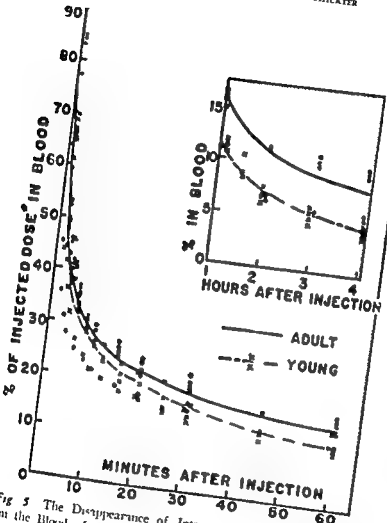

Fig. 5. The Disappearance of Intravenously Administered Calcium^{45} from the Blood of Adult and Young Rabbits

each curve represents the summation of at least four separate events. The mathematical expression for these events and their term by term comparison for the adult and the young animal is given in Table VII. The agreement of the rate constants of these four terms indicates that the mechanisms by which radiocalcium disappears from the blood are the same for adult and for young animal. However, the lack of agreement of the

compartment factors for the last two terms indicates that in removing the calcium from the blood the old animals use less of the third term mechanism and more of the fourth term mechanism than the young animals

TABLE VII

The Equations for the Disappearance of Radiocalcium from the Blood of Rabbits

Adult	$C(t) = 0.483e^{-1.36t} + 0.26e^{-0.177t} + 0.113e^{-0.0\ 4t} + 0.144e^{-0.00\ 4t}$
Young	$C(t) = 0.448e^{-1.76t} + 0.31e^{-0.\ 8} + 0.166e^{-0.0\ 6t} + 0.074e^{-0.00\ 6t}$

Term by term comparison of the rate constants b indicates that for all four individual processes the adult animals and the young animals are conducting business at rates of the same order of magnitude i e the nature of the business—the physicochemical event—is probably the same in each of the processes involved. While it may be true that the nature of the events is the same for the old and the young animal it seem that the quantities involved in at least the last two of the events are of different orders of magnitude Comparison of the A terms for example of the third component of the equations for old and for young animal indicates that some process in the old animals is getting less of the available business than a similar one in the young animals The identity of the b term indicates that both groups are handling the material in the same way Reciprocally the A factors of the fourth term indicate that the young animals are getting less material in this process than their parents but again the method of handling the material represented by the constancy of the b term is the same in both cases

The application of the type of data just presented is of utility only within the framework of its statistical validity

Analysis of the Fate of Radiocalcium in the Skeleton of Adult and Young Animals

The second general aspect of this study has been an attempt at an identification and correlation of the events which we know by their reflection in the blood may be in progress in other parts of the organism The uptake of intravenously administered radioactive calcium by the skeletal system as a function of time is illustrated for young and for old animals in Figure 6 In these studies the femur has been utilized as an index of calcium fixation by the bones It is clear from Table IX that the adult animals pick up less calcium in their bones than do the young but that both groups of animal employ the same mechanism for the bone uptake Further analysis indicates that this process is represented by the third term in the equation (Table VII) for the disappearance of radiocalcium from the blood

TABLE VIII

The Probable Error of the Rate Constants *(b)* and Compartment Factors *(A)* of the Terms of the Equation for Radiocalcium Disappearance from the Blood

	Adult Rabbits	Young Rabbits
A_1	0 483 ± 0 17	0 448 ± 0 175
b_1	1 36 ± 0 11	1 76 ± 0 25
A_2	0 26 ± 0 10	0 31 ± 0 09
b_2	0 177 ± 0 007	0 28 ± 0 11
A_3	0 113 ± 0 01	0 166 ± 0 02
b_3	0 024 ± 0 006	0 026 ± 0 002
A_4	0 144 ± 0 027	0 074 ± 0 014
b_4	0 0024 ± 0 001	0 0026 ± 0 0009

In Table VIII are listed the values of Table VII with a determination by the usual statistical methods of the probable error of the calculat ns It s apparent that the conclusio as to the parallel identity of all of the terms except the compart me t factors for the third and fourth term is justifable

Analys s of the respect ve curves indicates that they may be expre sed by the t rms in Table IX The rate con tant b for both young and old animals are the same Th s ind cates that the phys cal nature of the rad oactive calc um fixati n proces m ide t cal for the e grou s of animals On the oth r hand the compartment factor A is less in the ca e of the adult animals

Comparison f the terms in Table IX with the th rd terms previou ly deri ed from the curves of the d sappearan e of Ca^{45} from the blood s of interest The rate term b are of the same rder of magnitu le for the bl od disappearance event and the bone uptake event Th agreement is presumptive evidence for the identity of the b ne uptake pr cess with ts reflection in the bl od d sappearance curve Furth erm re the ratio of the adult/young compartment factors for the bl od d sappearance data is of the ame ord r a that for the femur p ck up data The difference between the compartment fact r for the adult and that for the y ung an mals i believed to repr sent me m a ure f the phy log c status of the skeletal sy tem of the animal

In the above description the variation in the compartment factor may be an expression of a physiol gic difference in bone metabolism due to age

compartment factors for the last two terms indicates that in removing the calcium from the blood the old animals use less of the third term mechanism and more of the fourth term mechanism than the young animals

TABLE VII

The Equations for the Disappearance of Radiocalcium from the Blood of Rabbits

Adult	$C(t) = 0.483e^{-1.36t} + 0.26e^{-0.177t} + 0.113e^{-0.0\ 4t} + 0.144e^{-0.00\ 4t}$
Young	$C(t) = 0.448e^{-1.76t} + 0.31e^{-0.\ 8t} + 0.166e^{-0.0\ 6t} + 0.074e^{-0.00\ t}$

Term by term comparison of the rate constants b indicates that for all four individual processes the adult animals and the young animals are conducting *business at rates of the same order of magnitude i e the nature of the business—* the physicochemical event—is probably the same in each of the process involved While it may be true that the nature of the event is the same for the old and the young animal it seems that the quantities involved in at least the last two of the events are of different orders of magnitude Comparison of the A terms for example of the third component of the equations for old and for young animal indicates that some process in the old animal is getting less of the available business than a similar one in the young animals The identity of the b term indicates that both groups are handling the material in the same way Reciprocally *the A factors of the fourth term indicate that the young animals are getting less* material in this process than their parents but again the method of handling the material represented by the constancy of the b term is the same in both ca

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Analysis of the Fate of Radiocalcium in the Skeleton of Adult and Young Animals

The second general aspect of this study has been an attempt at an identification and correlation of the events which we know by their reflection *in the blood may be in progress in other parts of the organism* The uptake of intravenously administered radioactive calcium by the skeletal system as a function of time is illustrated for young and for old animals in Figure 6 *In these studies the femur has been utilized as an index* of calcium fixation by the bones It is clear from Table IX that the adult animals pick up less calcium in their bones than do the young but that both groups of animal *employ the same mechanism for the bone uptake* Further analysis indicates *that this process is represented by the third term* in the equation (Table VII) for the disappearance of radiocalcium from the blood

TABLE VIII

The Probable Error of the Rate Constants *(b)* and Compartment Factors *(A)* of the Terms of the Equation for Radiocalcium Disappearance from the Blood

	Adult Rabbits	Young Rabbits
A_1	0.483 ± 0.17	0.448 ± 0.175
b_1	1.36 ± 0.11	1.76 ± 0.25
A_2	0.76 ± 0.10	0.31 ± 0.09
b_2	0.177 ± 0.007	0.28 ± 0.11
A_3	0.113 ± 0.01	0.166 ± 0.02
b_3	0.074 ± 0.006	0.026 ± 0.002
A_4	0.144 ± 0.077	0.074 ± 0.014
b_4	0.0024 ± 0.001	0.0026 ± 0.0009

In Table VIII are listed the values of Table VII with a determination by the usual statistical methods of the probable error of the calculations. It is apparent that the conclusion as to the parallel identity of all of the terms except the compartment factors for the third and fourth term is justifiable.

Analysis of the respective curves indicates that they may be expressed by the terms in Table IX. The rate constants *b* for both young and old animals are the same. This indicates that the physical nature of the radioactive calcium fixation process is identical for these groups of animals. On the other hand the compartment factor *A* is less in the case of the adult animal.

Comparison of the terms in Table IX with the third terms previously derived from the curves of the disappearance of Ca^{45} from the blood is of interest. The rate terms *b* are of the same order of magnitude for the blood disappearance event and the bone uptake event. This agreement is presumptive evidence for the identity of the bone uptake process with its reflection in the blood disappearance curve. Furthermore the ratio of the adult/young compartment factors for the blood disappearance data is of the same order as that for the femur pick-up data. The difference between the compartment factor for the adult and that for the young animals is believed to represent some measure of the physiologic status of the skeletal system of these animals.

In the above description the variation in the compartment factor may be an expression of a physiologic difference in lime metabolism due to a e

in the blood in the marrow. As a matter of fact, we can overlook the details and simply say that whatever the event is, it is represented in this way in the two groups of animals. In the adult as compared to the young animal, the ratio of these two compartments is clear: the adult has much less of a compartment available than does the young animal for this phase of his reaction.

Analysis of the Excretion of Radiocalcium in Adult and Young Animals

We have examined also the excretion of calcium[45] after it has been administered by intravenous injection in rabbits. In Figure 7 are plotted the data for the combined averaged urinary and fecal excretion of the animals in this study. Due to the fecal lag and the short time period over which a correlation with the blood data was being sought, the very early part of these curves may be considered to be a representation of urinary clearance. Analysis of the curves of radiocalcium excretion of adult and of young animal indicates that they may be described by the equation of Table V. For the short times under consideration, only the first terms of these equations come under scrutiny. The agreement in the rate constants again indicates that the mechanism of excretion for young and for old animals is the same. The difference between the compartment terms indicates that the old animal excretes more radiocalcium than the young. Further analysis indicates that calcium excretion is represented by the fourth term of the

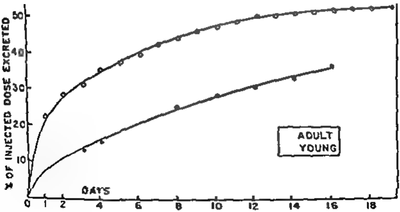

Fig. 7. The Combined Excretion of Intravenously Administered Calcium[45] in the Feces and the Urine of Adult and Young Rabbits

TABLE X

The Equations for the Combined Urinary and Fecal Excretion of Intravenously Injected Calcium [4] in Rabbits

Adult $\% E(t) = 0\ 15 e^{-0\ 0042t} + [illegible]29 e^{\ 0\ 00018t} + 0\ 56 e^{-0\ 000001\ t}$
Young $\% E(t) = 0\ 07 e^{-0\ 0013t} + 0\ 93 e^{-0\ 000016t}$

$\% E(t)$ is the percentage of the injected dose in the excreta at the end of time (t)

equation (Table VII) for the disappearance of radio calcium from the blood

Comparison of these values of Table X with the fourth term of the blood disappearance equations of Table VII is of interest It is apparent from the identity of the order of magnitude of the exponential terms that we are dealing with the same physicochemical event It is equally clear from the similarity in the ratios of old/young for the compartment terms of both blood disappearance and urinary excretion that the blood measurement has reflected the excretion process As a matter of fact it i possible to calculate from the fourth blood term what the excretion values ought to be if this term really measures the urinary excretion over the observation period The calculated and the observed data (Table XI) are in striking agreement

Armstrong Are we allowed to draw the inference again that there is no excretion of radiocalcium into the gut because your counts refer only to the urine? I refer here to the application of the fourth term to the urine

TABLE XI

The Calcium[45] Excretion of Rabbits after Intravenous Injection Calculated from the Blood Disappearance Data and Determined by Collection

	Adult	Young
	(%/24 hr)	(%/24 hr)
Calculated	14 4 ± 2 7	7 4 ± 1 49
Measured	15 0 ± 5	7 0 ± 4

Rubin No we can not All we can do is include the ... exchange if you want to call it that into ... term Our ... later work would indicate that this ... correct We ... that what goes into the gut goes ... quickly ... from actual test as a result of anoth... periment ... done We injected the calcium into ... that ... the sys tem ... uivalent ... ond ... as

being soft tissue distribution of calcium. Therefore in the urinary excretion study the fourth term does represent urinary excretion whereas the gut exchange is included in the earlier terms.

Studies of the Fate of Radiocalcium in Animals with Experimentally Altered Calcium Metabolism

We have felt that support for the conclusions developed above might be found from studies in which there was some deliberate experimental alteration in the calcium metabolism of the test animals. The deviations in the values derived from an analysis of the blood disappearance curves concomitant with such experimental changes should be evident.

CALCIUM LOADING EXPERIMENTS

In one group of experiments we have subjected the experimental animals to vigorous calcium loading by repeated daily intubation of calcium phosphate. After this procedure the blood disappearance curve of radioactive calcium[45] was determined in the usual manner (Figure 8).

The term values that resulted from the analysis of this curve (Figure 8) are compared to the normal third and fourth term values in Table XII. Several significant differences may be noted between these terms for the normal and for the experimental animals. The exponential value b of the third blood disappearance term of the normal animal is the same as that of

TABLE XII

The Effect of High Calcium Intake on the Blood Disappearance of Calcium[45] in Rabbits

$C(t)$ Normal $= 10.0e^{-0.03t} + 10.5e^{-0.007t}$
$C(t)$ Ca Excess $= 18.5e^{-0.04t} + 10.0e^{-0.039t}$

the experimental group. This is an indication that the physicochemical process by which calcium has been deposited in the bone in the two groups is identical. On the other hand the third term compartment factor A has been significantly increased in the experimental group and it may be concluded that the experimental procedure of calcium loading has increased the "area" of calcium deposition in the bone. In the same experiment it is noted that the exponential value b of the fourth term is different in the experimental group as compared to that in the normal animals. This may be

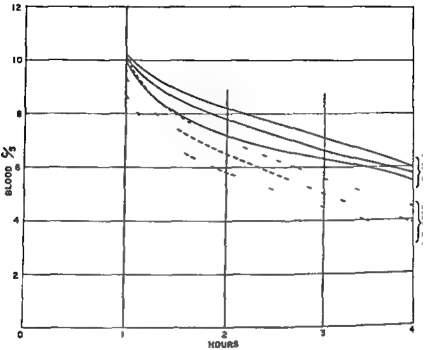

Fig 8 The Effect of Excessive Oral Intake of Calcium on the Disap pearance of Intravenously Administered Calcium[4] from the Blood o Adult and Young Rabbits

interpreted as a prediction that an altered mechanism of calcium eliminatio has come into play on a high calcium intake Likewise an increase in th compartment of excretion is noted

CALCIUM DEPLETING EXPERIMENTS

Attempts decisively to influence calcium metabolism in a negative direc tion also have been conducted In addition to the usual procedures we hav utilized chelating agents for this purpose These synthetic materials a will be described in more detail later have a pronounced ability to combin with calcium *in vivo* After the oral administration of one such material ethylenediaminetetraacetate EDTA we have determined the nature of th blood disappearance curve for intravenously injected radiocalcium As i

evident in Figure 9 the oral administration of the complexing agent has increased the blood disappearance of tracer calcium to a marked degree as a function of the administered dose of chelate

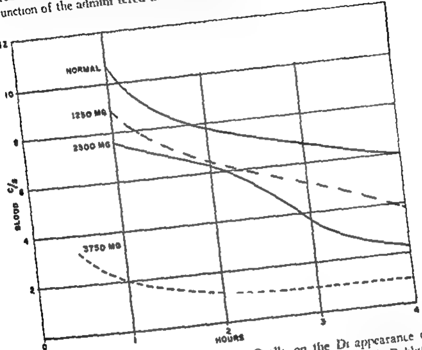

Fig 9 The Effect of Versene Given Orally on the Disappearance of Intravenously Administered Calcium[45] from the Blood of Adult Rabbits

Analysis of these curve indicated that the change in the distribution of the tagged calcium was due to an increase in the compartment value of the first two blood disappearance terms This finding implies that a new reservoir of calcium fixation had developed in the soft tissues Data obtained by distribution studies with carbon tagged chelate and supplied by Dr Harry Foreman [1] proved that orally administered chelating agent was not absorbed from the gastrointestinal tract Thus the hypothesis of fixation of calcium in a newly created reservoir has some support in fact

In conclusion we believe that we have demonstrated that by kinetic studies of the fate of radioactive calcium in the blood metabolic pool it is possible to obtain an accurate qualitative and quantitative measure of normal and of pathological calcium metabolism *in vivo*

Foreman H Vier M and Magee M to be published

Conference Discussion

Armstrong: Do you know whether the calcium that is chelated is exchangeable?

Rubin: Yes, we have studied that. It is completely and instantaneously exchangeable in solution. I cannot answer for the solid phases. But in serum it is completely exchangeable, or in aqueous solution it is. Radioactive calcium exchanges completely with the chelating agent. But this is not generally necessary. There are some chelates in which the metal is not readily exchanged for its isotope. Tagged iron and iron in hemoglobin do not exchange very easily. But calcium in this particular soluble complex does exchange completely.

Kramer: Does the chelate stay in the blood, is it excreted, or what happens to it?

Rubin: We will talk about that later, but to anticipate our discussion, it depends on the mode of administration. If the chelate is given intravenously, it is excreted very rapidly in the urine; if it is given orally, it is excreted in the feces up to 80 to 100 per cent. This has been shown with carbon tagged material.

Copp: Did you measure the fecal excretion in the adult rabbit?

Rubin: Yes, we have, and I have some charts on that point which I would be glad to show you.

Copp: Was it significantly greater than that in the young animals?

Rubin: Not significantly by our determinations. We did not find as much difference in the fecal excretion of the young as compared with that of the old animals as we did in the very early urinary excretion.

Copp: Well, that is quite different from the behavior of rats, where the chief path of excretion in the adult is in the feces. Excretion in urine is negligible. Remember, it is short term work you have been talking about, up to the first four hours in the rabbit, and there is a very considerable lag in fecal excretion in the rabbit.

Neuman: Have you had any difficulty with re-ingestion of fecal material by the rabbit?

Rubin: Do you mean mechanically?

Neuman: No, actually.

Rubin: No, because these animals were placed in metabolic cages in which the feces drop through immediately.

Neuman: Was the rabbit restrained? This is not my observation, but I have been horrified to learn—I do not remember who told me, but I think it

was Comar at Oak Ridge—that even in the metabolic cages the rabbit will re ingest feces during night feeding to a considerable extent

Rubin That is the reason why in the beginning we restricted our reference to the first four hours in order to simplify the situation as far as possible

Neuman We found with some insoluble substance administered to rabbits that the materials seemed to stay and stay and stay! It could not possibly be! The gut would have had to have been ten or twelve times as long as it actually is to account for this phenomenon by dilution It must have been due to re in tion

McCance I believe rabbits go in for this practice only at night and I don't suppose any experiments were carried out at night

Rubin We had three kibitzers and one workman he did not work at night [Laughter]

Shorr Has not the skin a considerable calcium content so that it should be analyzed separately?

Rubin We did not measure it separately

Armstrong Dr Shorr and I were discussing this point a moment ago Certainly within a few days after giving calcium intravenously to rats one can find several per cent of the injected dose in the pelt I have assumed and Dr Shorr says he disagrees with this that the radiocalcium got onto the skin mainly as a result of the habit of the rat of licking himself since the saliva has a concentration of calcium very near that of plasma calcium Do you want to debate this point Dr Shorr

Shorr It was my impression that there is a fair amount of calcium that is present in the skin and more in the older than in the younger skins Perhaps somebody can supply more specific data

Follis You refer to calcium in the skin not the hair that is to calcium in the epithelium?

Armstrong Yes but the calcium specific activity of the skin is very high for several days after the specific activity of the urine has fallen

Engel I think one has to consider that the calcium in the blood and elsewhere is essentially in equilibrium with the calcium in connective tissue and that it is the connective tissue of the skin which binds a certain amount of this injected or ingested calcium

Howard What kind of connective tissue of the skin

Engel Principally the ground substances but also the other negatively charged colloid in the skin

Armstrong What was the definition of ground substance at this conference last year?

Fremont Smith It was never agreed to

Engel I knew it! [Laughter]

Follis We presented three different definitions of collagen last year

Fremont Smith Dr Engel do you wish the electron microscopist s definition or the histologist s definition?

Engel It is really not necessary to define ground substance precisely at this time if you simply consider that there is a negatively charged colloid in the skin which has the ability to bind calcium There is negatively charged colloid distributed throughout the whole organism

Follis The mineral content of epithelium is higher than that of collagen Isn t that true?

Engel I don t know

Follis That is you can see more on microincineration in the epithelium than you can in collagen [8, 83]

Howard There is practically no calcium in tendon or fascia You cannot get enough to analyze

Shorr But it is true that there is an appreciable amount in skin

Howard Well I have Mitchell s data [84] here if you want them I did not include them in the talk The subject a 70 kilogram man was killed on the street Mitchell analyzed him from A to izzard and added it all up apparently and found 1 126 total grams of calcium in the body Ninety nine per cent all but 12 grams were in the bones and teeth and the skin contained 5 of these 12 I thought however that in the other tissues which contain calcium the calcium is exceedingly unlikely to be exchangeable

Armstrong Do nuclei contain calcium?

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[8] MacCardle R C Engman M F Jr and Engman M F Mineral Changes in Neurodermatitis Revealed by Microincineration *Arch Dermatol and Syph lol* 47 335 (1943)

[83] Scott G H The Localization of Mineral Salts in Cells of Some Mammalian Tissues by Microincineration *Am J Anat* 53 243 (1933)

[84] Mitchell H H Hamilton T S Steggerda F R and Bean H W The Chemical Composition of the Adult Human Body and Its Bearing on the Biochemistry of Growth *J Biol Chem* 158 625 (1945)

Howard Well that is what Mitchell finds by the microincineration technique

Copp We found definite amounts of radiocalcium in the skin in our rats too but it fell off at the same rate as the serum calcium We have assumed that this calcium was in the extracellular space

Armstrong I do not believe there is any calcium in the cells I think with red cells you certainly can show that all the calcium is in the plasma

Howard I cannot find any in red cells either but Mitchell with microincineration seems to identify some It certainly appears that way

Shorr One of the points that has always struck me in the use of warm blooded animals is the difficulty in differentiating between metabolic and physicochemical factors Would we not get some help if we used cold blooded forms in whom the rates of biological processes could be varied at will by changing the environmental temperature whereas physicochemical processes would change with temperature to a much less marked degree I hope this suggestion will send you scurrying for the giant Louisiana bull frogs which will permit you to draw enough blood for your chemical studies and allow for setting conditions which would vary metabolic rates

Neuman The nearest approach to the bullfrog that I can think of is contained in the studies by LaCroix[85] and by Arnold[86,87] I know that LaCroix has embedded bone sections in a plastic and then ground the plastic to an optically flat surface He dips this surface in radiocalcium and then exposes it to obtain a radioautograph He gets exactly the same histological picture of isotope distribution as when he injects the animal with radiocalcium At least *this* isotope uptake is essentially physicochemical Now this immediately brings up Leblond's[88] work which I am sure is still valid where he correlates the uptake of isotope with growth processes Have I confused everybody?

Copp I think both processes are active

Neuman Oh yes

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[85] LaCroix P Autoradiographs of Spongy Osseous Tissue *Experientia* 8 426 428 (1952)

[86] Arnold J S Progress Report Radioautography *Atomic Energy Report* ANL 4825 p 72 (1951)

[87] Arnold J S Calcium Metabolism of Growing and Mature Bone *Fed Proc* 11 5 (1952)

[88] Leblond C P Wilkinson G W Belanger L F and Robichon J Radioautographic Visualization of Bone Formation in the Rat *Am J Anat* 86 289 341 (1950)

Copp I do not think one or the other is exclusive

Follis There are three

Neuman Yes at least three

Armstrong I was very interested to see how Dr Rubin dissected the significance of the four rate controlled processes in his equations You can develop these equations in as many terms as you like and one may add terms until the equation fits the data to the degree that one wishes We used four terms he also employed four terms I was never willing to make any interpretation as to the processes indicated by the separate terms You may remember that in the work of Gellhorn[9] with sodium two terms were used to describe arterial plasma disappearance curves of injected radiosodium Actually the two terms were chosen on *a priori* grounds I think that one should have if possible *a priori* grounds for the selection of the number of terms in such equations Gellhorn s basis for the selection of two terms was the finding that the ratio of radiosodium in tissues to that in plasma was such that the tissues distributed themselves in two different general groups I think that your interpretation of the meaning of the third and fourth terms is quite an elegant process Would you speculate on what is described by the first term?

Rubin We deliberately have avoided talking about it We think that the first two at least seem to represent vascular mixing perhaps plus soft tissue distribution and beyond that we will not go We are beginning to pin down the second one by retrograde experiments of giving radioactive calcium into some soft tissue area and then measuring the blood uptake and we find for example that the rate of pickup in the blood of radioactive calcium given in the gut is identical with the rate for the second term This begins to argue to us that soft tissue distribution is represented by the second of the two terms

Armstrong Well I would like to say again that the rapidity with which these processes occur allows an opportunity for a change in the distribution and in the location of the mineral part of the bone as a result of physical processes which are biologically affected I hope by that statement to try to restore some harmony between Dr McLean and Dr Neuman

Copp I would like to mention an experiment that we carried out which I think supports both Dr McLean and Dr Neuman We found that if animals were given radiocalcium and were then restricted to a diet very low in phosphorus there was an immediate increase in calcium excretion

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[9]Gellhorn A Merrell M and Rankin R M The Rate of Transcapillary Exchange of Sodium in Normal and Shocked Dogs *Am J Physiol* 142 407 (1944)

and a negative calcium balance* This occurred within 24 hour and was very active within the first week or two before there were any histologic changes It is my feeling that this initial loss may have come from the surface calcium all through the skeleton because it occurred while the animal was still in very good health The only effect was a slightly negative calcium balance As has been pointed out previously there is relatively a large quantity of calcium which is superficial and not incorporated deep within the structure of the crystals

However after about two weeks when perhaps this source of calcium was exhausted then examination of the bones showed definite histologic evidence of resorption of bone matrix and bone and there were plenty of osteoclasts present in the section It may be that the superficial bone calcium provides an immediate reservoir of mineral but when this store is exhausted calcium and phosphate can be obtained only by complete biological destruction of localized areas of bone

Shorr Is that the only way that bone can be reduced in volume

Copp You must actually get some reduction in ash even by the first process There may be other changes but in this particular experiment we obtained histologic evidence of biological resorption of bone only after a period of two weeks and after quite a long period of negative calcium balance

Shorr So that accepting the criterion for example of osteoclastic activity as your first indication then that need not occur during the early phase?

Copp Of course there may have been something occurring before the histologic change was apparent

Follis That is probably a very crude estimation

Copp Bone resorption is apparent only after there is considerable change in the bone but prior to this there may be I think increasing biological activity and resorption in addition to a simple physicochemical process

Engel One could consider that all the phases of bone are in equilibrium with each other and that a change in any one phase necessarily implies a change in the other phases Present histologic methods of defining changes may be so crude as to make them not evident

Shorr In other words not in steps at all as this would imply?

Cramer C F and Copp D H Effect of Mineral Deficient Diet on the Excretion of Radiocalcium and Radiostrontium Am J Physiol 167 776 (1951)

Engel Yes

Neuman Yes that is true but I do not think you can say that all of the bone is in equilibrium

Engel But this work shows it too

Copp No I think that each part of the bone each of the different skeletal structures almost certainly has its own peculiar behavior For example we made a comparison between the behavior of incisors molars and typical long bones like femur in a condition of marked low phosphorus rickets where you get a loss of calcium Each of these skeletal elements behaved entirely differently

Neuman I would like to bring up not my own studies but some fairly unknown work that was presented at the last Federation meeting by Donald Buchanan[9] —

Armstrong On CO ?

Neuman Yes on CO He performed some elegant experiments in which he used an atmosphere containing radiocarbon in the form of CO maintaining a constant specific activity He used very young rats and very old rats In the young rats the specific activity of the bone carbon dioxide was essentially equal to that of the internal milieu but in the old animals it approached only 50 per cent equilibration indicating that half of the bones were out of the equilibrium Edelman presented the same picture with radiosodium in the adult dog where he felt that some 50 per cent of the sodium was not exchangeable[92]

Armstrong We got the same results as Edelman on the fraction of exchange

Neuman Yes your data support this point Kornberg[93] in England with radiosodium experiments on humans and on adult dogs obtained figures as low as 35 per cent The continuously growing animal such as the rat never approaches the state of maturity of the adult human I think we can say therefore that some figure between unity and 0 3 represents the general overall availability of the bone as you go from the very young to the very old These figures if you consider all the microscopic variability (which is a horrible hodgepodge) represent the overall average

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[9] Buchanan D L and Nakao A Bone Carbonate Turnover *Fed Proc* 11 19 (1952)

[92] Edelman I S James A H and Moore F D The Location and the Turnover of the Sodium of Bone *TRANS MACY CONFERENCE ON METABOLIC INTERRELATIONS* 4 240 247 (1952)

[93] Kornberg H A Unpublished results

Shorr But can't you disturb that by acidification?

Neuman Yes indeed as in rickets

Shorr So it is only relatively fixed

Neuman In rickets the bone is relatively available compared to a normal animal of the same age

Armstrong Dr Neuman as you know it has been shown many times that in the epiphyseal region of a bone even in an adult the calcium and phosphorus are more exchangeable than in the diaphysis Is this because of a difference in the anatomic location which is making the mineral phase more readily available to the body fluid or could you suggest another reason?

Neuman After harping on this matter of not recognizing ignorance I cannot get up and give the data I have because the results are only a correlation not an explanation For example it can be demonstrated that in the older bone there is no free available water Therefore these areas are isolated You cannot demonstrate that the new bone the epiphyseal bone has a much higher water content Yet the question still remains in this case whether the total water content cannot be accounted for on the basis of hydration of the crystals This provides a physicochemical explanation of the exchange data but why is there a difference in the water content? I am tempted to say this is biological

Armstrong Actually of course we never really explain very much We only frame a statement into other terms

Follis There is a difference in vascularity in the two regions that you speak of Dr Armstrong

Armstrong That is the one obvious factor which may be related to the point under discussion

ELECTRON MICROGRAPHY OF BONE[94, 95]

ROBERT A ROBINSON and MICHAEL L WATSON

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[95] This paper is based in part on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project Rochester N Y

[96] Ham A W Some Histophysiological Problems Peculiar to Calcified Tissues *J Bone and Joint Surg* 34A 701 728 (1952)

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200μ

ELECTRON MICROGRAPHY OF BONE[94, 95]

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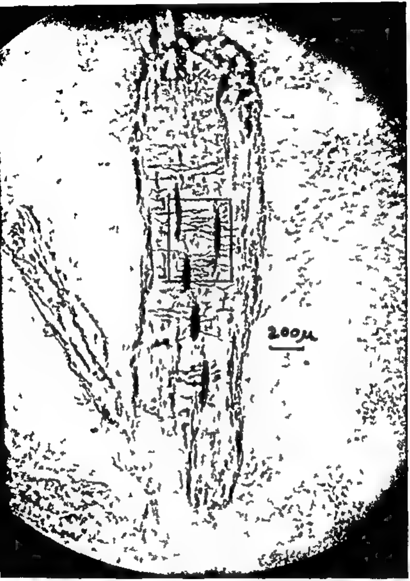

Fig. 10. Fragment of Bone with the Organic Component Removed by Autoclaving

Prepared by autoclaving the fragment for 4 hours at 27 pounds of pressure. Magnification 500 ×. In Fig. 11 the rectangle is shown in higher power.

[Reproduced by permission from Robinson, R. A.: An Electron Microscopic Study of the Crystalline Component of Bone and Its Relationship to the Organic Matrix. *J. Bone and Joint Surg.* 34-A, 389-434 (195[illegible]).]

of the crystals.[98] These bone crystals have been shown to be tabular in habit. When obtained from this autoclaved material these crystals appear to have average dimensions of 500 by 250 by 100 Å. The surface area of a gram of crystals having these dimensions, considering their specific gravity to be about 3, was calculated to be 106 square meters.[99] These crystals gave a powder x-ray diffraction pattern typical of the apatite crystal lattice.

The Physical Characteristics of Collagen Fibers from Decalcified Bone

It was possible to decalcify the bone by placing thin shavings in [illegible].125 normal trisodium Versenate solution buffered at pH 7.0 with Na_2HPO_4–KH_2PO_4. Treatment of thin bone shavings in this solution using constant agitation required about 24 hours for decalcification. The material was then agitated in distilled water in a Waring blendor. The collagen fibers of the extracellular matrix of bone were thus revealed. These fibers when dried on the specimen screen of the electron microscope were shadowed with uranium. Figure 14 shows a small bundle of the fibers obtained by the method just outlined. Some amorphous material appears to remain in the central area from which the fibers protrude. The fibers are seen to be thicker at the doublet bands than between.

Collagen fibers obtained as were those in Figure 14 were stained with phosphotungstic acid. In Figure 15 one can detect the five inter-period small spacings in each major 630 to 640 Å period of the human bone collagen.

Crystal and Collagen Fiber Relationships in Hyaluronidase Treated Human Rib Cortex

When fresh human rib cortex was incubated with streptococcal hyaluronidase at pH [illegible] for three hours and blended, it could be more easily disintegrated in the Waring blendor than could untreated bone. However, inorganic bone crystals were found still clinging to many of the fibers in such a sample of bone matrix (Figure 16), and one finds the crystals are laid down along the fiber at intervals of about 630 to 640 Å. As noted above the crystals are much thinner in one direction than in the other two. In general, it was noted that the broad surface of the tablet-shaped crystals was parallel to the direction of the fibers. This was particularly evident

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[98] Robinson, [illegible] A., and Bishop, F. W.: Method of Preparing Bone and Tooth Samples for Viewing in the Electron Microscope. *Science* 111: 655 (1950).

[99] Robinson, [illegible] A.: An Electron Microscopic Study of the Crystalline Inorganic Component of Bone and Its Relationship to the Organic Matrix. *J. Bone and Joint Surg.* [illegible]A: 389-434 (1952).

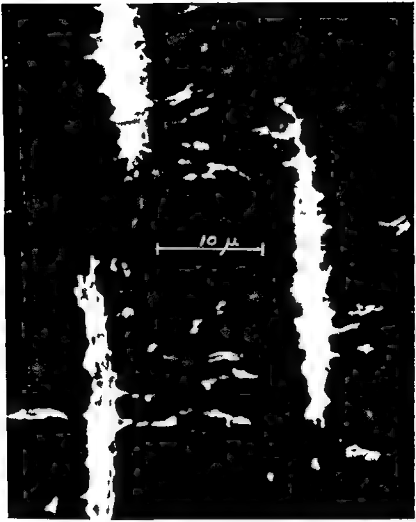

Fig 11 Fragment of Bone with the Organic Component Removed by Autoclaving High Power Magnification of Rectangle in Fig 10

Magnification about 5000 × The structures are identified in Fig 17

[Reproduced by permission from Robinson, R A An Electron Microscopic Study of the Crystalline Component of Bone and Its Relationship to the Organic Matrix *J Bone and Joint Surg* [illegible] A 389 434 (1952)]

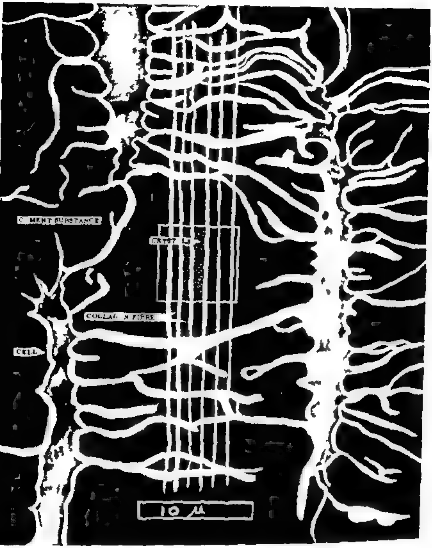

Fig 12 Fragment of Bone with the Organic Component Removed by Autoclaving High Power Magnification of Fig 11 Retouched to Show the Outlines of the Canaliculi and Lacunae

Magnification about 500 × The collagen fibers have been represented diagrammatically in the extracellular matrix pace by white vertical lines The approximate field of the electron microscope working at about 7000 × has been outlined in the center of the figure and in this the crystal are represented by white dots The many canaliculi should be noted for in livin bone these form the system of inter connecting canal by which the cells lying in the mid t of the calcified matrix maintain contact with nutrient blood vessels [7]

[Reproduced by perm ssion from Rob nson R A An Electron Microscopic Study of the Crystall ne Component of Bone and Its Relationship to the Organic Matrix *J Bone and Joint Su g* 34 A 389-434 (1952)]

Fig 13 Electron Micrograph of Autoclaved and Blended Human Bone Showing the Bone Crystals
Magnification about 64000 ×

where the crystals were on edge and appeared as thin black lines These black lines always paralleled the *long* axis of the fiber where the crystals were close to the fiber

Crystal and Collagen Fiber Relationships in Undecalcified Bone

At this point in our investigation concerning the submicroscopic struc

Fig 14 Electron Micrograph of Versene Decalcified and Blended Human Rib Cortex Showing Shadowed Bone Collagen Fibers
Magnification about 24 000 ×

ture of bone one of us developed tissue sectioning techniques[100, 101] so that

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[100]Watson M L A new Microtome for Thin Sectioning for Electron Microscopy *Quarterly Technical Report of the University of Rochester Atomic Energy Project* UR 205 67 71 (1952)

[101]Watson M L. A Method for Complete Extraction of Embedding Material from Tissue Sections for the Electron Microscope *Quarterly Technical Report of the University of Rochester Atomic Energy Project* UR 205 60-66 (1952)

Fig 15 Electron Micrograph of Versene Decalcified Blended and Phosphotungstic Acid Stained Collagen Fibers from Human Rib Cortex

Magnification about 24 000 ×

[Reproduced by permission from the original of *Figure 1* in Robinson R A and Watson, M L Collagen Crystal Relationships in Bone as Seen in the Electron Microscope *Anat Rec* 114 387 (1952)]

thin sections of Versene decalcified and undecalcified bone could be cut [2] (see Figures 17 through 28) These sections demonstrate the dense collagen web in the extracellular matrix of bone some of the features of the interfibrillar space and the arrangement of the bone crystals in relation to the collagen fibers

[When Figure 22 was projected a photographically negative lantern slide was used and it elicited the following question]

Follis What are those dark spots?

Robinson When you compare Figure 22 to the usual light microscope picture of bone (Figures 10 11 12) those dark spots (seen as light collagen free areas in Figure 22 in the text) are so-placed that they can be interpreted as canalicular apertures in other words they represent the canals which interconnect the lacunae of the osteocytes in the calcified dense fibrous matrix of living bone

In sections of undecalcified bone such as that seen in Figure 23 one can detect the underlying collagen fiber direction since the crystals are laid down in rows on the underlying matrix These rows of crystals lie at right

---

[2] Robinson R A and Watson M L Collagen Crystal Relationships in Bone as Seen in the Electron Microscope *Anat Rec* 114 383-410 (1952)

Fig 16 Electron Micrograph of Fresh Human Bone Incubated in Hyaluronidase and Blended

Magnification about 62 500 × The bands at 640 to 630 Å are made by bone crystal *adherent to the fibers*

[Reproduced by permission from Robinson R A An Electron Microscopic Study of the Crystalline Component of Bone and Its Relationship to the Organic Matrix *J Bone and Joint Surg* 34 A 389 434 (1952)]

angles to the direction of the fibers in the underlying matrix The fibers are not apparent in such sections probably for two reasons a) *The fibers* are partially masked by the inorganic crystals which are not removed of course in undecalcified preparations (The cement substance *may also help* to mask the fibers in undecalcified sections) b) There is such a great density difference between the inorganic bone crystals and the less dense organic collagen fibers that when one reaches adequate exposure of the photographic plate in the electron microscope for good crystal *definition the* film is markedly underexposed for fiber definition

The fiber direction does not show up directly in these undecalcified sec*tions except at the periphery* where a fiber is occasionally seen encrusted with crystals (Figure 28) Such areas on the periphery confirm the fact

Fig 17 Llectro M crograph of a Se t o of Verse Dc al he l O mc Ac d F e l and n Butyl Metl acrylate En bedded H man Rib Cortex

Magn fi o about 000× compa e w F ures 11 and 12 In the cen e a on f a bone a n w h en nan of ce l uctu e can be o e ed Th ough n he m x a e sma l ape u e n e p e ed as he s es of cana ul wh h pen t a ed e e y d n e co la er fib r ma x between the cells

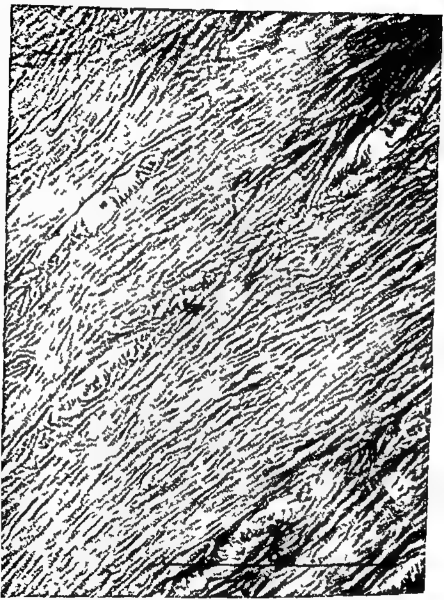


Fig 18 Electron Micrograph of a Section of Versene Decalcified Osmic Acid Fixed Human Rib Cortex High Power Magnification of a Section Similar to Fig 17

Magnification about 16 000 × Note the canaliculular apertures and the typical collagen large period banding

[Reproduced by permission from the original *Figure 5 in* Robinson R A and Watson M L Collagen Crystal Relationships in Bone as Seen in the Electron Microscope *Anat Rec* 114 397 (1952)]

Fig. 19. Electron Micrograph of a Section of Versene Decalcified Formalin and Osmic Acid Fixed Human Femur Cortex.

Magnification about 8400 ×. The final preparation was lightly shadowed with uranium and the typical banding of the collagen fibers was thus demonstrated. A bundle of parallel running collagen fibers was cut almost perpendicular to its long axis; in other words the bundle was coming up through the section whereas the others were lying nearly parallel to the plane of the section.

[Reproduced by permission from the original of Figure ... in Robinson, R. A. and Watson, M. L.: Collagen-Crystal Relationships in Bone as Seen in the Electron Microscope. *Anat. Rec.* 114 383 (1952).]

that the collagen fibers run at right angles to the rows of inorganic crystals in these undecalcified sections of comparatively parallel fibered bone of human rib cortex.

The Physical Characteristics of Bone Crystals

In electron micrographs such as that shown in Figure 24 one can actually see the individual crystals. The crystal length was measured in such

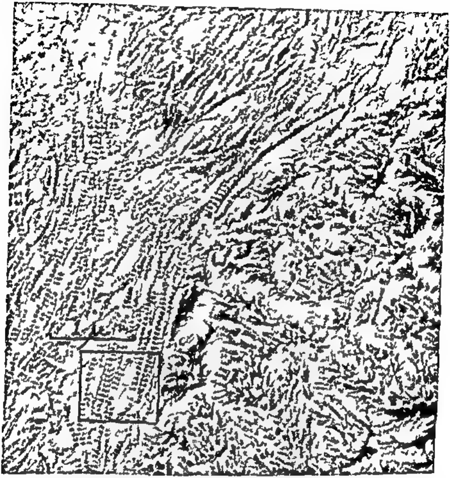


Fig 20 Electron Micrograph of a Section of Versene Decalcified Formalin and Osmic Acid Fixed Human Femur Cortex High Power Magnification of a Portion of Fig 19 Showing the Cross Cut Bundle of Collagen Fibers

Magnification about 30 000 × The space between the fibers represents the region that formerly contained the hydrated cement substance The inorganic crystalline component in the living bone apparently extends out into the interfibrillar space from the periphery of the fibers at the levels of the doublet bands In Fig 21 the rectangle is shown in higher power

sections and the measurements were grouped into families differing in length by 50 Å A plot of the number in each family versus the median length gave a curve with two peaks one at 180 Å was four times as high as the other at 400 Å It is possible that many of the smaller crystal plaques

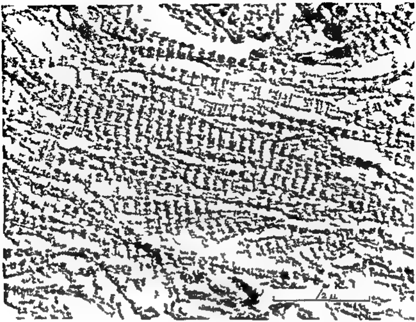


Fig. 21 Flect o M ograpl of a Se t n of Ver ene Deca c fied Fo al a d Osn \ d F el H n a Fem Cortex H gh Power Ma n ficat on of l e tan e n F 20

Mag a b u 78 000 \ Co n b n e g b dge b ee h hb a e l o he d ub ba d o h ag n a be

[R p odu d p m o f om h o a of F 4 Fo n F A and Va M L C g n C a R a n h p n B ne a S n he El n I o ope 4 P 114 9 952]

we e ra f la ge r s al r ken du g e t on n O th las a el e e t at a mature bone r s a tl ese e t ons of h man r b cor x a a le t of 400 Å the e t o of the o lagen a Many f the al a e f u d t be s and ng o e e a d mea ureme s at the j ot a l the shado a t on ere nd dual plaques e found nd a e a th ck es of 25 to 50 Å (ee F re 25) Tl e w dth of tl e plaques e e d f ult to de e ne l u many of them l y n the p ane of tl e ect on appea e l to ha e a l h co paral le o or sl ghtl less than the r length In he cas of the 400 Å cr s a s there a usuall a defin te *lo g* ax and tl e d h was bout 250 to 500 Å It l ould l e no e l pa t c la ly that al mo t one of the cr stal n t e bo e ons of the cortex of l un a r l we e o er 400 Å n length Tl ese d nen on are sl gl tly less than tl o e

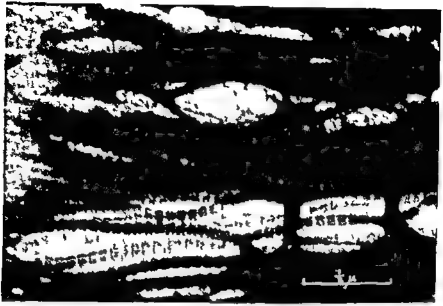


Fig. 22. Electron Micrograph of a Thin Section of Osmic Acid Fixed but Not Shadowed Bone.

Magnification about 78,000 ×. What appears to be connecting bridges between adjacent fibers at the levels of the doublet bands are observed.

[Reproduced by permission from the original of *Figure 5* in Robinson, R. A., and Watson, M. L.: Collagen Crystal Relationships in Bone as Seen in the Electron Microscope. *Anat. Rec.* 114: 399 (1952).]

which were obtained from specimens of autoclaved human cortical bone.[89] The preparation methods may slightly alter the inorganic crystal size both in the tissue sections and in the autoclaved preparation. However, variations in crystal size in specimens from various parts of the skeleton may occur.

We wish to emphasize not so much the absolute dimensions of the crystals as the fact that the bone crystals observed in all methods of preparation, whether fresh blended, autoclaved, glycol ashed, or sectioned, were much thinner in one dimension than in the other two. This means that the surface area to volume relationship was always very large.

Comparison of Undecalcified and Decalcified Areas of Human Rib Cortex

Sections of a piece of partially decalcified human rib cortex, as shown in Figure 26, revealed areas where the boundary between the decalcified region

and the ndecalc fied re, on could be seen Decal ficat on has spread n roughl c rcular areas around tl e ca al cul The harp bo ndary l et een the ful y al fied and decalc fied matr x should be noted The great d ffer en c ■ dens ty bet en tl undecalc f d and tl e decal hed reg o s ind cates l y n crograph of the undecalc fied bone sect ons fa ed to how collagen and sho d onl cry tal It sl ould l e note l also tl at tl e Ver ene may ren o e ot only the norgan c cr stals but also tl e terfibr llar cement sul ta ce Tl s ■ ent sub ■ ce t ı tl o gl t n a l el to ma k the ollagen fibers n undecal fied preparat o ı

Foll s Is the c n ent s l tance polysaccnar de?

Rol son Tl e polysac har de prote ■ complex called ce nent substance apparentl f ll up the interfibr llar spaces n tl e extracellular matrix of conne t ■ t sues It s my pre ent oncept that the r stal l e n tl s cement sub tan e n tl e bone matri or project out nto t from the per pl ery f the f bers

Sect ons of part ally d calcified r b vere stud ed at h gher magn fica t ons as seen n F g re ?7 At the boundary of tl e decalc hed reg on there are po nts wl re botl tl e cry tals and the underl ng collagen f bers can be seen At tl ese pots t appears that the crystals l ■ at tl ■ loublet bands and to a much le ser extent or not at all between the ets of loul lets Tl s strong ■ dence that tl ere s a l as c assoc at on between tl e r g on of the ollagen band and the overly ng crystals

It shoul be me t o e l at tl s po nt tl at Han* ha sl o n tl at vl ere l ne re orpt on tak n, pla e *n zo* one doe not see c lagen fibers unma l ed as they are d ng the *vitro* deca c ficat n of l o e Instead the cr tal ce nent s bsta ce and collagen ma d appear mul aneou l o the cement subs ance and cr tals ma l relea ed j st pr or to the d s appearance of the collag ■ fibers I otl er ord *v o* d r g bone resorr on f l ers d appe aln o t as rap ll as the cen ent bstan e and cr tal Unl ke th *zo* proce *z tro* decalcifica o w th Ver ene lea ■ tl e col age fiber mo ■ or less ntact so that tl e are read l recog n zabl n se t o s n tl e electron m cro ope

The Flectron D ffract on Pattern of Undecalc fied Human Rib Cortex

Us ng a se t on of undecalc hed human r b cortex n wh ch tl e fiber d rec on was *know (not only b the d re t o of tl e fe fil* ers at the edge f the sec o but a o b tl e d rect o of tl e row of cr tals tl e sec t on) an electron d ffract on pattern was ade (ee F gure 9)

The ele tron d ff act on pat ern (F gure 29) tl at g e by tl e bas c

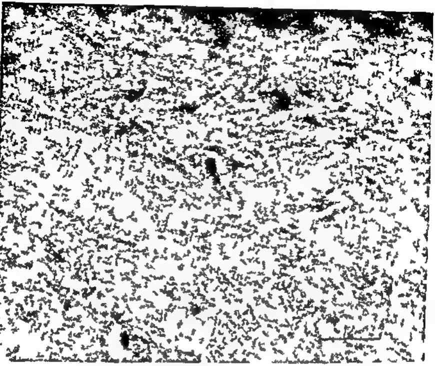


Fig *23* Electron Micrograph of a Sect on of U decalc fied O m Ac d F xed a d n But l Methacr late Embedded Human P l Cortex

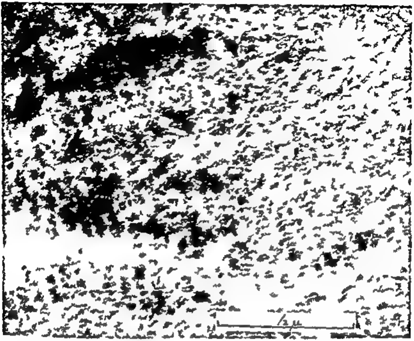
Magn fi on abou 34 500 × The of no an b ne y als a e see They a e se le e t on a r glt angle to t und l co agen fib s of t e na T e o vs e ur abou e e y 630 Å

[Reprodu ed by pe m f om he o g nal of *F g* *6* Rob n o P A a l Wa son M L Co agen C r al Rela n h p n B See he l e n M c o cope *A o* *R* 114 401 (19 ?]

calc um phosphate apat te var ety of crystal structure and s con pat ble ith hydroxyapat te Th n lar to the x ray d ffract on pat erns wh ch ha e sugge ted that hydroxyapat te s the most probable central crystal latt ce of bone cr stals The d ffract on r ng n F gure ?b lo ntens ty ar at ons or arc ng The arc ng n the *00?* and *00l r os* s

S ul le R Conce n g the F ne St u u e of Bone *Fo a d G b d l g ns a l* 57 31 ?64 (1937)

[6] B andenbe ge E and S h nz H R Co ce n ng the Na u e of Ca c fica n Man and An ma and he Beha or of Ino gan c Bo e Sub tan e e M n F D ea es f Ma *H* *M d A a (S pp 16)* 12 1 63 (1945)



F g 24 Electron M crograph of n Se t on of Unde al fied O n Ac d F xed and n B tyl Methacr late Embed led Hun an R Cortex H h Magn ficat o of a Port on of F g 23

A gn a n abou 110 000 ×. The elec on opaque ows a e f und o be mad up of he no gan bo e c y al Almo none of he e al n e f uman b o ex ce ded 400 Å n e h

[Reprodu ed by pe m on om e o p na o f Rob on R A and Wa on M L Co g n C y al Rela n h p n B ne as Seen he L M ope A I 114 403 195]

o ented w th respect to the e t o n a a wh ch allo s one to o cl de hat the c a es of the cr al un t cell o the a erage l e paral lel r nearly o to the a erage d rect on of the col gen f ler a es

F gure 30 g es one a cle nat c o cept of the un t cell of hydroxyapat te ts d me s o look g at the u t cell from the top (*A*) look g at t fro the s de (*B*) and the relat ve po t ons of the calc um ph o phate and hy l ox l group gs the un t cell latt ce (*C*) The or entat o of the axes of un t cel n a tablet haped bone crystal sho n n F g re 31 and 32 The c ax s of the n t cell thought to l e or ent d t the l ron l

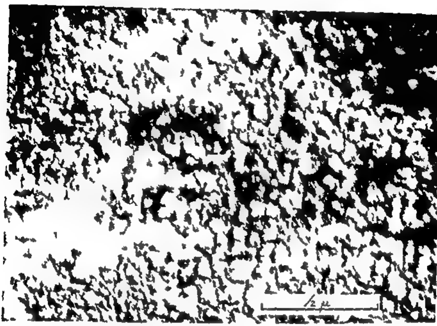


Fig 25 Electron Micrograph of a Section of Undecalcified Osmic Acid Fixed and n-Butyl Methacrylate Embedded Human Rib Cortex Similar to That in Fig 24 but Shadowed

Magnification about 94,000 ×. The section was shadowed at an angle of 7:1 with uranium to determine the thickness of the inorganic crystal

[Reproduced by permission from the original of *Figure 8*, Robinson, R. A. and Watson, M. L.: Collagen Crystal Relationships in Bone as Seen in the Electron Microscope. *Anat Rec* 114: 403 (1952)]

crystal surface for two reasons: (a) the broad surface appeared to be parallel to the collagen axis (Figure 16), and (b) the collagen axis and the c axis of the apatite unit cell were shown to be nearly parallel on the average (Figures 28, 29).

The Electron Diffraction Pattern of Calcium Phosphate Crystals

It was felt that the studies of bone crystals did not establish with sufficient definition the crystal habit or the relation of the *c* axis of the unit cell to the *long* axis of the crystal. Accordingly, studies of basic calcium phosphate crystals precipitated from water solutions were undertaken. These crystals had the habit of elongated tablets or laths[65] (see Figure 33).

Watson, M. L., and Robinson, R. A.: Unpublished data (1953).

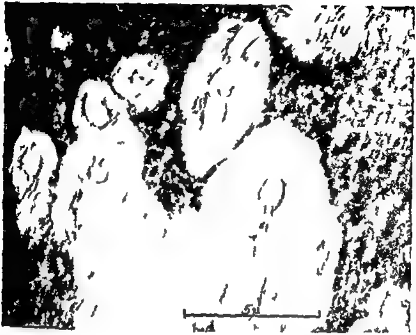


Fig 26 Electro M rographl of n Sect o of a Verse e P rtl Decal c ed O e Acd F e l n d n I tyl Methacrylate I n l ed led Bone

Magn fi a about 11 500 × No h a e s f d al ed ma x abou e ama a ap u e h ha p bounda y be een ca fed and d al fied m a x and he ag n fib d e n ela on o he y l ou

[P p od d by p m m f om t e o g nal of F u e 13 R b n R A an Wa s M L Co ag n C a R la on l ps n Bone as S e n he E on I o op I 114 409 (195]

It a pos l le to or e t the m lat l aped crys al n a early p rallel array o a pla t e replica of a l ffract on grat ng (ee F gu e 34)

Tl e ele tro d ffr ct on pa tern shown n F re 35 as prepared fro tl e or nte l las cal u n pl o plate apat te crystals sce n F g re 34 Cal ulat ons l n d o tl e arc ng angle nd cate tl at tl e c ax s l es tl t o degrees of tl e *lo g* cry tal ax s an l that therefo e t l es parallel to the b o d surface of tl e cr stal Otl er features of th ve t ga on are d u ed el e l ere s

I s ro g Tl s n eans tl at you do not ha e to or e t tl e c ax s n each cry tal I t yo l a e to or ent all crystal n the same ay doesn t t

Fig 27 Electron Micrograph of a Section of a Versene Partly Decalcified Osmic Acid Fixed and n Butyl Methacrylate Embedded Bone High Magnification of a Portion of Fig 26

Magnification about 49 000 × The crystals apparently lie at the doublet band of the collagen and to a much lesser extent or not at all between the sets of doublets

[Reproduced by permission from the original of *Figure 14* in Robinson R A and Watson M L Collagen Crystal Relationships in Bone as Seen in the Electron Microscope *Anat Rec* 114 409 (1952)]

Robinson The smaller crystals may not be big enough to show a discernible *long* axis but the *c* axes of their unit cells are of course oriented in relation to each other in the apatite lattice In bone these unit cells of the apatite crystals are oriented preferentially in relation to the collagen fibers of the organic matrix

The Orientation of Crystals and Collagen Fibers in Human Bone

I might emphasize that the bone crystals seen thus far in the tissue sections of human bone we have are about 400A long and we have observed what we believed to be crystals that were so small that they were at the lower limit of the resolving power of the electron microscope In the case of crystals around 180A in length and less no *long* axis was apparent Al

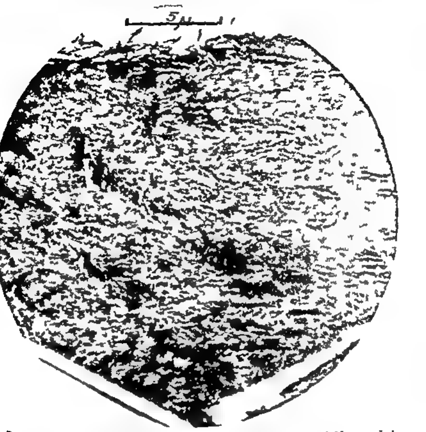


Fig 28 Electron M crograp l ot a Sect o of L kcal f e l Hun a I l Co tex

Ma n f a on about 6 900 ×
[R p oluc d by pern n f om the org n l of *F g e 9* R b nson, I A and Wa son M L Co ag n Cry tal I ela o l 1 Bo e as S e n he Elec on Mu oscope I a I 114 405 (1952)]

tho gl tle sn aller crystals were aln o t fo r t es a pre lc t a tle larger rysta the l ne se t o ol er ed tlese sect o s ga e a stron 1 e l n na t are ng wh cl ndicated tl at tle c ax s of the t cll nearl pa all led or paralleled the l rectio of tle colla e fibers Il ner a far as o e can tell tl at it s ot a me l an cal fact r wl ch la or e ed tle un t cell axes w tl tle u lerl ng collagen n atr x s nce n o t of tle cr tal d d n t l ave a *lo g* ax s Son e cl en cal or pl ys co l en cal relat o l 1 mu t or ent tl e olecules of the cr tal n t cells w tl tl e f tle col agen f e s of tl e org n c matrix

The collagen fibers show a defin m n ole ular or e tat n l l g es a

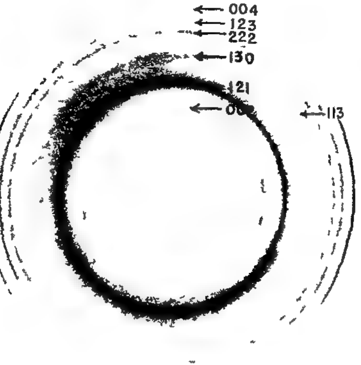


Fig 29 Electron Diffraction Pattern of the Section of Undecalcified Human Rib Cortex Shown in Fig 28

The diffraction rings show intensity variation or arcing The arcing in the 002 *and 004 rings is oriented with respect to the section in a way which allows one to* conclude that the *c* axes of the crystal unit cells on the average lie parallel or nearly so to the average direction of the collagen fiber axes

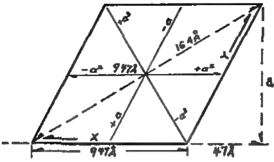
[Reproduced by permission from the original of *Figure 10 in* Robinson R A and Watson M L Collagen Crystal Relationships in Bone as Seen in the Electron Microscope *Anat R c* **114** 405 (1952)]

→

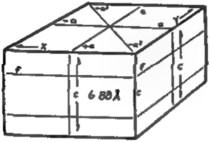
Fig 30 Schematic Drawings of the Unit Cell of Hydroxyapatite to Show Its Dimensions and the Relative Position of Its Constituents

The dimensions of the unit cell are shown in *A* and *B* and the relative position of the calcium (Ca) the phosphate (PO) and the hydroxyl (OH) groups in the unit cell are shown in *C*

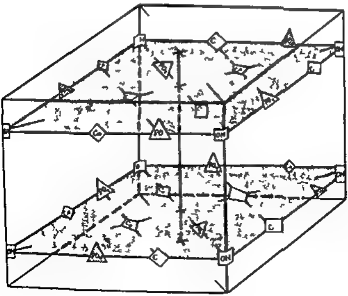
[Reproduced by permission from Robinson R A An Electron Microscopic Study of the Crystalline Component of Bone and Its Relationship to the Organic Matrix, *J Bone and Joint Surg* **34 A** 389-434 (1952)]



A



B



C

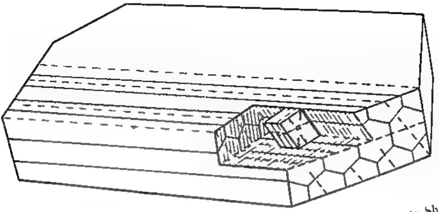


Fig 31 Schematic Drawing of the Crystal of Hydroxyapatite to Show the Orientation of Its Unit Cells to the Broad Crystal Surface

From investigation of synthetic basic calcium phosphate crystals evidence is available that the *long* axis of the crystal and the *c* axes of its unit-cells are parallel This drawing should be compared with that in Fig 32

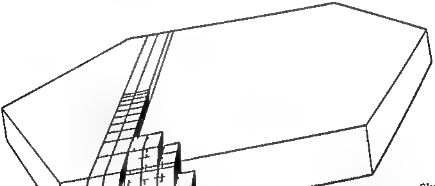


Fig 32 Schematic Drawing of the Crystal of Hydroxyapatite to Show the Orientation of Its Unit Cells to the Broad Crystal Surface

From investigation of synthetic basic calcium phosphate crystals evidence is available that the *long* axis of the crystal and the *c* axes of its unit cells are parallel. This drawing should be compared with that in Figure 31

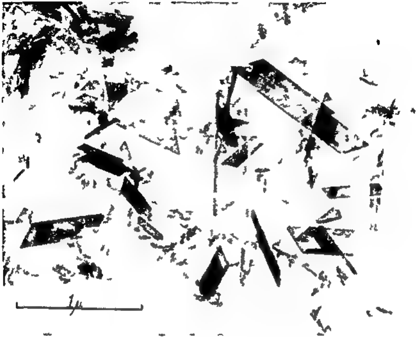


Fig 33 Electron Micrograph of Synthetic Basic Calcium Phosphate Crystals

Prepared by precipitation from a water solution Magnification about 74 000 ×

x ray diffraction pattern and since the fibers appear before the crystals in osteoid they may act as the orienting factor and crystallizing nucleus for inorganic bone crystals We have shown a correlation between the doublet bands of the collagen fibers and the position of the overlying apatite crystals in human bone and this observation adds support to the argument that the inorganic molecules depend for their orientation in bone matrix on the underlying collagen fibers

Implications of the Organic Inorganic Relationships in Bone

It has been reported that the bone crystals in autoclaved samples from

---

[1] Bear R S The Structure of Collagen Fibrils *Advances Prot in Chem* 7 69 160 (1952)

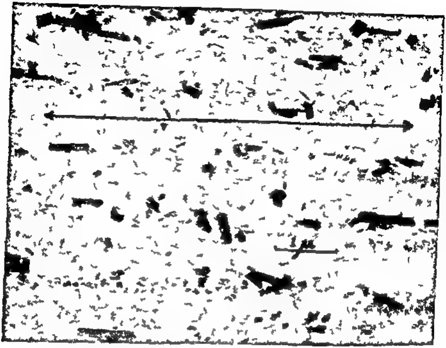


Fig 34 Electron Micrograph of Synthetic Basic Calcium Phosphate Crystals such as Those Shown in Fig 33 Oriented in Nearly Parallel Array on a Plastic Replica of a Diffraction Grating

Magnification about 22 000 ×

humans have an average dimension of 500 × 250 × 100 Å[20] In the sectioned material from the outer cortex of the human rib the average crystal size was smaller[10] and therefore it would appear that between 100 and 250 square meter of exchangeable surface area existed per gram of human bone crystal, assuming that the bone crystals have the specific gravity of hydroxyapatite which is about three This may be true in samples of varying crystal size from which all the organic matrix has been removed But as one can see in the bone sections the crystals are intimately associated with the organic matrix and the ability of atoms to migrate from the blood plasma to the bone crystal surfaces must be governed by the freedom with which these inorganic atoms can migrate through the organic matrix

Table XIII is a simplification of a table which was previously published[22] It shows the relative volumes of organic matter plus water and of inorganic bone crystals to the total volume of bone at different ages and sites The

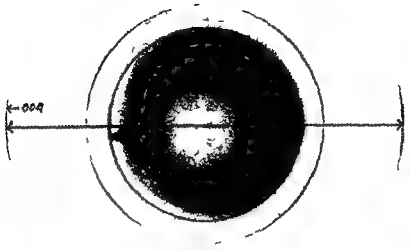


Fig. 35. Electron Diffraction Pattern of Oriented Synthetic Basic Calcium Phosphate Crystals in Fig. 34

The 002 and 004 rings show arcs. These arcs are bisected by the *long* axes of the crystals [illegible] pattern of the diffraction grating

crystals at best comprise only a third of the volume. In young bone the organic matter plus water occupies about 77 per cent of the bone volume. In the older bone these constituents occupy about 72 per cent of the volume, and in the most compact bone such as the cortex of the diaphysis of the femur the organic matter and water occupy about 67 per cent of the total fresh volume. The water volume is 30 per cent or more in young cancellous bone and drops down to 20 per cent or less in older compact bone of cortex. The organic material remains relatively constant while the inorganic material somewhat increases in volume as the bone becomes older.

TABLE XIII

The Relation of the Volumes of Organic Matter Water and Inorganic Bone Crystals to the Total Volume of Bone at Different Ages and Sites

Constituent	Cancellous bone				Compact Bone	
	Young		Old			
	Volume (Approx.)	Relation to Total Volume (Approx.)	Volume (Approx.)	Relation to Total Volume (Approx.)	Volume (Approx.)	Relation to Total Volume (Approx.)
	(cc)	(%)	(cc)	(%)	(cc)	(%)
Water	18	30	11	20	8 10	15 20
Organic Matter	29	47	28	52	25 27	52 57
Organic Matter plus Water	47	77	39	72	35	67
Inorganic Bone Crystals	14	23	15	28	17	33
Total in 100 Grams	61	100	54	100	52	100

Amprino and Amprino and Bairat [8] have shown that the rate of microscopic reconstruction is higher throughout life in spongy bone of the epiphysis and of metaphysis than in compact bone of the diaphysis Amprino and Engstrom [09] showed by historadiographic technique of ground sections of bone that minerals are unevenly distributed in its matrix i e the calcium content is 5 to 20 per cent lower in bone tissue of recent formation By radioautographs of bone sections taken from animals that had been given labeled phosphates Engfeldt Engstrom and Zetterstrom

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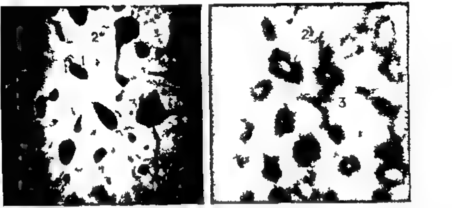
Amprino R The Structure of Bone Tissue Regarded as an Expression of the Difference in the Velocity of Growth *Arch Biol Paris* 58 315 330 (1947)

[8]Amprino R and Bairat A Processes of Reconstruction and Reabsorption in the Compact Substance of the Bones of Man Research upon a Hundred Cases from Birth until Late Life *Ze tschr f Zellforsch* 24 439 511 (1936)

[09]Amprino R and Engstrom A Studies on X Ray Absorption and Diffraction of Bone Tissue *Acta Anat* ... 15 1 22 (1952)

[110]Engfeldt B Engstrom A and Zetterstrom R Renewal of Phosphate in Bone Mineral II Radioautographic Studies of the Renewal of Phosphate in Different Structures of Bone *Biochim et Biophys Acta* 8 375 380 (1952)



F g 36 \ ra M crorad ograph (left) and I ad oauto ra}l (r ght of a Sect on of Cor ex from the Femur of a Dog to Sl o v the Up ake of Ra l oact e Pl ospl a e

Magn fi a n abcu 40 × T e dog wa g en 10 m of d a t e pho p a e h ee da b f e a fi ng *1* a young Ha e a s n —an o d Ha e s an y m and *3* b ue su u g a e o p a y The up ak h gh e you Ha e an y em n he ld Ha e e and zero n bone ue u ound ng a e o p on ca y

[Rep xlu d b pe m on f m Engfe d II Eng om A nd Ze e om R Pe a of P pha n B ne M ne a s II R au og ap S ud s of he P ewal of I o a es D ffe en S u u of Bo e a *B p a d a* 8 375 380 1952]

l wed a gr ter ra l oact v t re e t la d du osteo l tl an l ler [ry bone t s ue n tl e cortex of d g bone (F gt re 36) B u n l n e t on l} pe l n Ca^{45} as calc m chlor de t as found by Ampr no a l LaCro x [3] that ra l oact tv a l gl er n tl e recentl forme l an l l s calc fie l tr ct res tl an the full calc hed ol ler stru t res Wl e tl e organ n atr x as ren o ed l gl col a h ng or nc nerat on al o e 500 C tl e a reg of tl e l one se n t k } Cr [3] at a o e e l u for rate

P tt r H tl k a ect l ee l t n ke tl autora l o g pl s?

Amp no P A o ad g apl An. l f he D bu n of Labeled Ca um a l Pho p u Bon *Lxp ri* 8 ?0 2 (195)

A p P I n I pe m n on e F xa n *In I* of Pa Ca n Se n s of I *Exp a* 8 380 38 (19

L C o x I Au rad graph of Spongy Bone T ue *L p* *nt a* 8 4 (1)

Robinson They were made with ground bone sections of about 25 to 50 micra thickness I did not make these sections of course This picture is taken from the paper by Engfeldt Engstrom and Zetterstrom [11]

Such investigations have emphasized that a) throughout life part of the bone is newly formed and part is old b) in the very young animals most of the bone is new and has a lower calcium content in relation to its total volume and a higher water content c) in mid life the new bone that is formed roughly equals the amount of bone resorbed d) in old age bone destruction exceed bone formation and new bone is minimal in amount relative to the total volume so that the bone assumes microscopically a Swiss cheese appearance in senile osteoporosis [109] and e) the amount and physical state of the extracellular organic bone matrix is associated in some way with the availability of the inorganic bone crystals for isotope exchange

The Effect of Water Content and of Age on the Availability of *Bone Calcium for Isotope Exchange*

Combining the material from the various sources mentioned above and the data upon which the Table XIII are based it would appear that most of the bone in young animals is new with a water content over 20 per cent while in older animals there is a much smaller proportion of new bone the majority of the bone or the largest part of the bone probably having a water content of 20 per cent or less In the new bone with the higher water content radioactive calcium and phosphorus exchange both *in vivo* and *in vitro* has been shown to take place readily By the same techniques in older bone such exchange is noted to be markedly less So that as the skeleton ages it shows a decreasing exchange as well as a lower water content [4, 11]

The point to be emphasized is this there may be a potential surface area on human bone crystals of between 100 and 250 square meters per gram of crystals but these areas are either not available for exchange or are only partially available for exchange It is proposed that when the water content of the organic bone matrix decreases to some critical level around 20 per

---

[11] Neuman Wm F The Nature of the Mineral Phase of Bone *Chem Reviews* in press (1953)

[115] Neuman cites the following references to experimental work relevant to this concept The percentage of bone available for P^{32} exchange is nearly 100 per cent in *young rats* according to C Leblond D Buchanan using C^{14} found that in adult rats the exchangeable fraction of bone dropped to about 50 per cent I S Edelman using Na^{24} in adult dogs found the exchangeable fraction varied from 35 to 52 per cent In an adult human H A Kornberg using Na^{24} found that the bone available *for* exchange was 30 per cent

cent the number of crystal surfaces available for exchange with the blood becomes very small In senile states more and more of the bone comes to have a water content of 20 per cent or less and then of the total area of bone crystals less is available for exchange [16]

Conference Discussion

Armstrong I would say to Dr McLean and to Dr Neuman that whereas the composition of the bone salt cannot be stated and is a very complex material we certainly see here that its *biological orientation and pattern* is quite regular at last we have something regular Does anyone wish to draw Dr Robinson out further?

Shorr Do you think the crystals are related to or are held in the ground substances or are they stuck in some chemical fashion to the connective tissue as in fibrils? I was not clear as to what you wanted us to infer

Robinson You are asking me if the crystals are in the collagen or around the collagen?

Shorr Yes You mentioned ground substances I did not know what your interpretation was

Robinson Well the discussion went on here last year about that

Shorr Yes I recall it

Robinson The collagen fibers are very durable structures Collagen fibers can be taken out of the mammoth tusk which has been lying about for some twenty thousand years and still be seen in the electron microscope as a recognizable entity Apparently around these fibers there is interfibrillar material and perhaps there is some of this same material between the component fibrils of the collagen fiber It would appear as though these fibers were not always arranged in exact spatial register one to the other but that there was some interconnecting link between the fibers at the most nearly adjacent 630 A period doublet bands as brought out in the osmic stained and shadowed electron micrographs It is these bridges and if you conceive of it in a three dimensional view these interband planes that connect the fibers It is at these same levels that the crystals appear The crystals seem to be oriented so that the c axis of their unit cells—and if they grow big enough their *long* axis—is parallel to the collagen fiber That is our picture of it at the present time

Shorr But do they just float in a medium around them or are they actually fixed tightly in this position?

Ampr 13 R. Factors that Regulate the Structural Remodeling of Bone *Arch Inst Sc Pr I* **31** 708 4 (1946)

Robinson: They were made with ground bone sections of about 25 to 50 micra thickness. I did not make these sections, of course. This picture is taken from the paper by Engfeldt, Engstrom and Zetterstrom.[110]

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The point to be emphasized is this: there may be a potential surface area on human bone crystals of between 100 and 250 square meters per gram of crystals, but these areas are either not available for exchange or are only partially available for exchange. It is proposed that when the water content of the organic bone matrix decreases to some critical level around 20 per

[114] Neuman, Wm. F.: *The Nature of the Mineral Phase of Bone. Chem. Reviews,* in press (1953).

[115] Neuman cites the following references on experimental work relevant to this concept. The percentage of bone available for P^{32} exchange is nearly 100 per cent in young rats according to *C. Leblond.* M. Buchanan using C^{14} found that in adult rats the exchangeable fraction of bone dropped to about 50 per cent. I. S. Edelman using Na^{24} in adult dogs found the exchangeable fraction varied from 35 to 5[illegible] per cent. In an adult human, H. A. Kornberg using Na^{24} found that the bone available for exchange was 50 per cent.

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Shorr But do they just float in a medium around them or are they actually fixed tightly in this position?

Ampe... R Fa... s that R gulate the Structural Remodeling of Bon *A* *h* *d* ... *R* *l* ... 38 4 (1946)

Robinson The crystals do not "swim around." They are oriented to the organic matrix on a molecular level. It is not possible at this time to state definitely whether this orientation factor is directly or indirectly exerted by the fiber on the inorganic molecules. One can point out that in many places "bridges" between fibers seem to connect their doublet bands. These "bridges" are observed in the decalcified sections of bone to lie in the same planes where the rows of crystals are seen in sections of undecalcified bones.

At present we do not know just where the fibers end and the cement substance begins on a chemical level of organization. Do these "bridges" represent particularly well polymerized muco-polysaccharide protein molecules, or do they represent protein molecules which *in vivo* belong to the collagen system and still connect the fibers laterally, even after the rigors of tissue preparation? If the "bridges" represent regions where the fibers were particularly close and the muco-polysaccharide protein cement substance was more resistant, then a muco-polysaccharide protein "bridge" exists and one might suppose that the crystals form in the "cement substance" between the fibers and that they may be oriented to the collagen molecules indirectly. In other words, an orientation might be imposed on them by "oriented" cement substance molecules which were in turn oriented (perhaps before the bone crystals formed) by adjacent collagen fibers. However, if the "bridges" represent protein molecules belonging to the collagen network, then one might suppose that the crystals formed on the protein molecules of the collagen network and were directly oriented by the molecules that formed part of that system. They would then project into the interfibrillar area from the periphery of the fiber.

EQUILIBRIUM OF CALCIUM AND OTHER IONS IN CONNECTIVE TISSUES[117]

MILTON B ENGEL NORMAN R JOSEPH and HUBERT R CATCHPOLE

From the Departments of Dental Therapeutics and Orthodontia of Chemistry and of Pathology The Colleges of Dentistry Pharmacy and Medicin Uni rsity of Illinois Chicago Illinois

Armstrong I think Dr Engel s work on the interaction of ions with the connective ti ue would fit in well at this point

Engel The work that I am going to report has been done in collabora tion with Dr Norman R Joseph and Dr Hubert R Catchpole my asso ciates at the University of Illinois

Electrometric Studies of Alterations in the State of Constituents of Connective Tissues

Various types of connective tissue vary greatly in their water electrolyte and colloid content and they may exhibi rather striking changes under the influence of hormones in a very hort interval of time We have studied these alterations n the state of connective tissues using an electro metric method and the changes in hydration and plasticity have been cor

[117]This work was supported by grants f om the American Cancer Society rec m mended by the Committee on Growth of the Nat onal Research Council from the Graduate Schocl Univers ty of Illinoi and fro n the Medical Research and Develop m nt Board Office of the Su geon General Department of the A my Contract No DA 49 007 MD17

[1] Gersh I and Catchpole H R The Organization of Ground Sub tance and Base m nt Membrane a d Its Significance in Ti sue Injury Disea e and Growth *Am J Anat* 85 457 (1949)

[19] Joseph N R Engel M B a d Catchpole H R Interaction of Ions and Connective Tissue *Biochim ica et Biophysica Act* 8 575 (1952)

Loeb L Suntz ff V and Burns E L Changes in the Nature of the Stroma i Vagina Cerv x and Uteru of the Mou e Prod ced by Long Co tinued Injection of Estrogen and b Advan ing Age, *A n J Canc r R s* 35 159 (1939)

Catchpole H R Jo eph N R and Engel M B The Action of Relaxin on the Pubic Symphy s of the Guinea Pig Studied Electrometrically *J Endocrinol* 8 377 (195_)

Joseph N R Engel M B and Catchpole H R Home sta is in Con ective T i t be publi hed

related with the density of immobile anionic charges of connective tissue colloids In the interaction of the ground substance with cations there are indications that the immobile anions may participate in cation exchange reactions [119, 123, 124]

Follis Will you define what you mean by colloid?

Engel The colloid refers to all immobile charged macromolecules of the tissue and I will not be any more specific than that I will say that it includes the mucoproteins of ground substance it includes collagen it includes all of the extracellular proteins as well as the intracellular proteins I would like to leave it general at this point if I may

With the electrometric method a liquid junction is made in the connective tissue under study and the potential is measured From such data the density of colloidal charge can be calculated

The circuit may be represented as follows

Hg	Hg Cl	KCl	NaCl (0 15M)	Subcut Tissue	Experimental Tissue	Solution I or II	KCl	Hg Cl	Hg

Solution I refers to isotonic NaCl (0 15M) with which the baseline potential is measured Solution II denotes either a one tenth dilution of the isotonic saline (0 015M NaCl) with which the dilution potentials E or E are determined or a solution of 0 01M CaCl plus isotonic NaCl which is used to equilibrate the tissue with calcium ions For the reference junction a #22 hypodermic needle was inserted subcutaneously in the abdomen or thigh It was filled with isotonic NaCl and connected to the calomel half cell with a saturated KCl bridge A short # ? needle filled with isotonic NaCl was inserted into the connective tissue to be studied as for example the pubic symphysis of the guinea pig the tibial epiphysis of the rabbit or the sex skin of the monkey

The circuit was completed through the other calomel half cell and the baseline potential was determined This is usually close to zero millivolts When the isotonic NaCl at the experimental site is replaced by a one tenth dilution of NaCl a dilution potential E is obtained

In dense connective tissue positive potentials of 20 to 30 millivolts are found an example is the pubic symphysis of normal guinea pigs When this tissue loosens as during pregnancy the potentials fall to zero or negative values approaching a limiting value of —12 3 millivolts which corresponds to the liquid junction potential of purely aqueous solution The changes in potential reflect changes in colloid water and electrolyte of the tissue [119, 121, 122]

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[123] Meyer K and Rapport M M The Mucopolysaccharides of the Ground Substance of Connective Tissue *Science* 113 596 599 (1951)

[124] Neuman W F Boyd E S and Feldman I The Ion binding Properties of Cartilage *TRANS MACY CONFERENCE ON METABOLIC INTERRELATIONS* 4 100 112 (1952)

If one applies the theory of liquid junction potentials as developed by Henderson,[1] an equation is derived which indicates the amount of the immobile negatively charged colloid in the tissue. This can be simplified to the expression

$$E_d = -12.3 + 21.1x \quad (1)$$

where E_d refers to the number of millivolts and x is the number of equivalents of colloid. From data obtained through these potentials it is then possible to calculate the number of equivalents of negatively-charged colloid in various connective tissues. It is an interesting observation that in loose connective tissue the value of x is relatively low, and in tight dense connective tissue (as, for example, in bone) the value of x is high.

The Effect of Calcium on the Colloid of Connective Tissues

In determining the effect of calcium on the colloid, a solution which contains 0.01 molar calcium chloride in 0.15 molar NaCl is first used to perfuse the tissue we wish to study (for example, the epiphysis). A baseline reading is established. The calcium solution is then replaced with the dilute NaCl (0.015M), and we find that our dilution potential is lower than the original, indicating that the immobile negatively charged colloid, x, has been decreased.

This may be expressed by a modification of Equation 1:

$$E_d = -12.3 + 21.1x \quad (2)$$

This implies, then, that the calcium combines with a part of the colloid, neutralizing some of the immobile charges.

We have found, in general, with the 0.01 molar calcium chloride, that approximately 0.6 of the colloid remains unbound and that 0.4 is bound by the calcium. If one measures the colloidal charge in a number of tissues of the monkey (for example, in the sternum, in dentin, in gingiva, or in skin), one gets values of negative charged colloid (0.168, 0.147, 0.095, 0.072, respectively) (Table XIV).

Handler: What are the units of x?

Engel: These are in equivalents.

Handler: Per milliliter of material?

Engel: Per liter, or per kilogram if the density is close to one.

Now, upon perfusing with 0.01M calcium, the colloidal charge density of cartilage drops to 0.090, the dentin to 0.102, the gingiva to 0.057, and the skin to 0.044. In general, a ratio of about 0.6 can be established between the modified colloidal charge and the original charge (Table XIV).

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[1] Henderson, P.: Thermodynamics of Liquid Cells. *Z. Physik. Chem.* 59, 118 (1907); 63, 3[illegible] (1908).

TABLE XIV

The Electrochemical State of Connective Tissues

Tissue	Density of Colloidal Charge* (x)	Modified Density of Colloidal Charge† (x)	Ratio $\left(\frac{x}{x}\right)$
MONKEY			
Sternum	0 168	0 090	0 54
Dentin	0 147	0 102	0 69
Gingiva	0 095	0 057	0 60
Skin	0 072	0 044	0 61
RABBIT			
Epiphysis	0 144	0 099	0 69
Skin	0 071	0 048	0 67

*Equivalents per liter

†After equilibration with 0 01M CaCl + 0 15M NaCl

Armstrong When you speak of the epiphysis as the site into which you put your needle do you mean the epiphyseal cartilage?

Engel I do mean that because on dissection it appears fairly clear that the needle has penetrated into the epiphyseal cartilage

Armstrong If I have understood what you have done here you really have set up concentration cells—am I correct?

Engel Yes

Armstrong And you observe differences in the concentration of the ions which you attribute in some cases to removal by the colloid?

Engel We observe a fall in the dilution potential after calcium which we attribute to the binding of the calcium by the colloid

Armstrong How can you be sure that it is the colloid which causes your results? How do you know it is not the mineral phase that is capturing some of the ions? You must have an answer since I am sure you must have considered this point

Engel Because the type of dilution potential that we get (which is a positive dilution potential) can be derived only in the presence of a negatively charged colloid After you introduce the calcium salt the dilution potential falls It approaches more nearly to water values which implies combination with immobile anions If the calcium were merely combining with another mineral phase it would not affect the concentration of x which is to say the dilution potential would not be altered

Armstrong I see That is clear

Follis What kind of reading do you get with just a blood vessel?

Engel We have not done that but one would probably get a reading which would be very close to the one for water because the concentration in equivalents of immobile anion in the blood should be quite low Our experience has been that in connective tissues that are very loose we get dilution potentials very close to those one would expect in water

Shorr Have you added any other divalent ions

Engel On one or two occasions we have tried magnesium and we got similar effects but we are not in a position to report on that now in other words magnesium will also combine with the colloid

Parker If there is anything like bound phosphate you would get the same result wouldn't you if it had a free radical

Engel If there were bound phosphate

Parker Yes

Engel Meaning an immobile phosphate?

Parker Yes

Engel I believe so Ling at Johns Hopkins has attributed to bound phosphate a similar effect in muscle Do you know of that work?

Sobel Yes roughly

Engel Our electrometric studies were conducted on rabbit epiphysis and rabbit skin with similar results (Table XIV)

Figure 37 is a curve showing the relationship between the dilution potential before and after perfusing with calcium solution and the slope is approximately equal to 0 6

A Dissociation Constant for Calcium in Connective Tissue

From data of this sort we have attempted to calculate a dissociation constant of the kind that McLean and Hastings established for blood

The calculations are as follows

Ling G Selective Ionic Accumulation in Muscle Cell *J ... Pr ...* 11 95 (195)

McLean F C and Hastings A B The State of Calcium in the Fluid of the Body I The Conditions Affecting the Ionization of Calcium, *J ... Chem* 108 285 ... (1935)

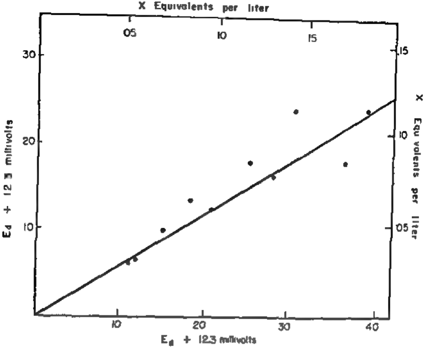

Fig 37 The Correlation of the Dilution Potential (E_d) and the Modified Dilution Potential (E_d') for the Normal Connective Tissues of the Rabbit and the Monkey

The abscissae E_d + 12.3 millivolts and x equivalents per liter—the estimated density of negative colloidal charge and the ordinates E' + 12.3 millivolts and x equivalents per liter—the estimated density of negative colloidal charge after equilibration with isotonic NaCl + 0.01 M CaCl

$$\frac{(Ca^{++})\ (x^{=})}{(Ca_t)} = K \quad (4)$$

Equation 4 is modified to compensate for the Donnan effect which occurs in these tissues due to the presence of negatively charged colloid and the expression for ionic calcium of tissue becomes

$$(Ca)\ \left(1 + \frac{x}{0.15}\right)$$

(Ca++) refers to the concentration in equivalents of applied calcium and x is the modified colloidal change *In the intact animal it would coincide with the* value of serum ionic calcium From this expression it can be seen that in dense tissues where x approaches 0.15 the calcium ion concentration may be double that of blood *Equation 4* then becomes

$$\frac{(Ca)\left(1+\frac{x}{0.15}\right)x}{(1-x)} = 10^{1.6} \quad (5)$$

We have found that the value we get for the constant is $10^{1.62}$. The constant that was established by McLean and Hastings for serum ($10^{-2.2}$) would seem to indicate a somewhat stronger affinity of blood proteins than connective tissue colloids for calcium. The blood proteins are then about 12 percent saturated with calcium at the physiological level of calcium ions, that is 0.00125 molal. In loose connective tissue the colloidal charge may be regarded as approximately 0.01 equivalents which is only about 5 percent saturated with calcium. In dense connective tissue ($x = 0.15$) the colloid is approximately [illegible] percent saturated. I tried to bring out earlier that one has to conceive of all the connective tissues, even the loosest, as containing a certain amount of calcium. A part is bound to protein and a part exists in an ionic state. These forms may be regarded as being in equilibrium. The equilibrium distribution of the mobile anions and cations is given for loose and dense connective tissues in Figure 38.

A Nomogram for Calcium and Colloid in Connective Tissue

From these data and from the estimated equilibrium constant a nomogram (Figure 39) has been constructed to represent the state of the tissue in relation to two independent variables, namely blood calcium and colloidal charge in the tissue. The nomogram relates the bound calcium to the colloid in the tissue and to the other ions. In loose connective tissue, of course, the amount of bound calcium is relatively smaller than it is in dense connective tissue.

The upper and lower dotted lines of the nomogram constitute an envelope which defines two extreme states, the loose state and the tight state of connective tissue. These states are in equilibrium with the blood calcium and they are also in equilibrium with each other. With a nomogram of this sort, once one has determined x and once one has a value for the blood calcium level, it should be possible—and I must call your attention to the fact that this is just a first approximation—to calculate, for example, the sodium in the tissue, the amount of bound-calcium, the ionic calcium which is in equilibrium with that, and adding the two calcium values together, the total calcium of the tissue, exclusive of course of the crystal or apatite phase.

In case you are wondering about how the levels for sodium are obtained, they are derived by an approximation formula for the Donnan correction. The sodium concentration would be approximately equal to

$$0.15 + \frac{x}{2}$$

Follis: What are examples of loose and tight connective tissue?

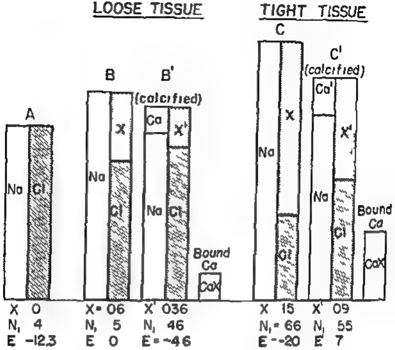

Fig 38 The Distribution of Ions as Estimated from the Dilution Potentials (E_o and E_d)

A—isotonic NaCl solution in which the sodium ions carry 40 per cent of the current (x = 0 N_t = 0.4 E_d = −12.3 mv) *B* — *loose connective tissue* (x = 0.06 N_t = 0.05 E_d = 0 mv) *B'*—loose connective tissue after equilibration with isotonic NaCl + 0.01 M $CaCl_2$ (x = 0.036 N_t = 0.46 E_d = −4.6 mv) *C*—dense connective tissue (x = 0.15 N_t = 0.66 E_d = −20 mv) and *C'*—dense connective tissue after equilibration with isotonic NaCl + 0.01M $CaCl_2$ (x = 0.09 N_t = 0.55 E_d = 7 mv)

Engel Loose and tight connective tissue rather than calcified?

Follis Yes

Engel A loose tissue would be a tissue like monkey sex skin which is slightly edematous and a tight tissue would be a tissue like the connective tissue of the pubic symphysis of the guinea pig or bone or cartilage—the cartilage of the epiphysis

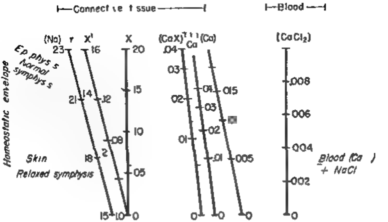

Fig 39 Connective Tissue Nomogram Showing the Equilibrium Distribution of the Cations in Relation to the Colloidal Density of the Charge

All concentrations are expres ed in equi alents per l ter The connect e ti sue scal s *(Na+)*—the ionic sod um —the Donnan ratio *x*—the density of the col l idal cha ge at equ librium with the calc um ions of the perfusate *x*—the init al d nsity f th collo dal cl a ge as observed with calc um free solut ons *(CaX)*—the b und calcium *(x x) (Total Ca)*—the total calc um and (Ca++) —the ionic cal ium The blood scale *(CaCl$_2$)* —the concentration of calcium chl ride of the equilibrating perfusate in equival nts per liter The phys ologic level ■ ind cated

Summary A Two Phase Equilibrium of Colloid Water and Electrolyte in Ground Substance

I should like to conclude my presentation by pointing out then that the state represented by epiphysis sternum and dentin (at the upper limit) and skin (at the lower limit) envelop a family of converging lines corre ponding to all the intermediate physiological states and that homeostatic variations of the colloid r at constant osmotic pressure but variable water and electrolyte content have been explained in terms of a two phase equilibrium in ground substance Accordingly all lines converging from various values of *r to the Donnan equilibrium dialysate represent homeostatic* states

Conference Discussion

Armstrong Do you know whether Dr Eichelberger s data on cartilage compo ition can be fitted into your nomogram?

Engel Before I came here I went to the library for that reference but

I have not had an opportunity to read it.[128] But I do know that last year Edelman[130] and his associates presented data on bone before this group, and their values for sodium, for example, would fall at the high extreme of the nomogram. If you take the low extreme, the values for monkey sex skin, which have been determined by Ogston, Philpot, and Zuckerman[131] a number of years ago, also fall within the values calculated for sodium and chloride on the nomogram, and I would assume the values for calcium also would approach that calculated on the nomogram.

Armstrong: I have one other question I would like to ask. Do you think our explanation of the calcification process, which has been developed over a period of two or three years at this Conference, to explain why tissues that ought to be calcified became calcified and those that do not get calcified *fail to become calcified on the basis of the accumulation of calcium in the* cartilage or bone matrix prior to calcification, is too much simplified? If I have understood your discourse correctly, we now will have to abandon this explanation because you have shown that connective tissue which does not calcify *will accumulate calcium.*

Engel: I think there are many sides to this problem, and one that I have neglected to mention because I do not know very much about it is: what happens to phosphate? While all of these tissues do contain calcium, much of the calcium exists as a proteinate; there is another phase in equilibrium with that, the apatite phase. I suggested this morning that one might conceive of a system in which there are multiple phases, a heterogeneous system of phases in equilibrium, that the apatite phase is in equilibrium with the calcium colloid pha■ and that both are in equilibrium with blood and tissue

---

[128] We have since had an opportunity to recalculate Eichelberger's data in order to convert her values of sodium and calcium to milliequivalents per kilogram of tissue. Her values were *originally expressed as milliequivalents per 100 grams of dry weight.* Taking the water content of cartilage as 75 per cent, the figures for sodium and total calcium agree well with the values for cartilage shown on the nomogram. The ratio of cartilage s[illegible] to serum sodium yield values for the Donnan ratio, the ionic calcium of cartilage, and for r, the density of immobile colloidal charge. The difference between total calcium and ionic calcium represents bound calcium. All these *results also agree* well with the nomogram. The values of r, ionic calcium, and bound calcium yield a value of K, the equilibrium constant, with which our own is in substantial agreement.

[129] Eichelberger, L., Brower, T. D., and Roma, M.: Histochemical Characterization of Inorganic Constituents, Connective Tissue and the Chondroitin Sulfate of Extracellular and Intracellular Compartment of Hyaline Cartilages. *Am. J. Physiol.* 166: 378 (1951).

[130] Edelman, I. S., James, A. H., and Moore, F. D.: The Location and the Turnover of the Sodium of Bone. *TRANS. [illegible] CONFERENCE ON METABOLIC INTERRELATIONS* 4: 240.

[131] Og[illegible] Philpot, J. [illegible] S.: Observations Related to the Sw[illegible] Skin in R[illegible] *J. Endocrinol.* 1: 231 (1939).

fluid When I say equilibrium I refer to the chemical potential of these various components the calcium chloride and the various calcium phosphates

Harrison Is the concentration of the calcium ion uniform throughout the system?

Engel I neglected to state that in our view the extracellular fluid and the ground substance are part and parcel of the same system If you consider this to be at least a two phase system in which you have a colloid rich water poor pha e in equilibrium with a water rich colloid poor phase which includes the extracellular fluid then it is all one physicochemical system In homeostasis each pha e maintains a constant compo ition and constant chemical potential While the chemical potentials of all diffusible components are constant throughout the system the electrolyte concentrations of the water rich and coll id rich phases differ from each other

Handler What was the actual range of calcium concentration?

Engel Most of our measurements were made at 0 01 mols but we also used a more nearly physiologic oluti n namely 0 005 mols and got similar results

Howard What are the quantitative aspects of the calcium content in tissues that are not cartilage and bone? For in tance did you do tendon? What would be the calcium content of a certain weight of tendon?

Engel From the n mogram (Figure 39) I will take a guess at it and then maybe somebody can check it

Howard Well we have analyzed it and cannot find any I was just wondering—

Engel I would expect some to be there

Howard But in such amounts that we could not measure it by the ordinary technique?

Armstrong What are you asking about?

Howard The quantitative a pects of calcium in the skin connective tissue the tendon and similar sub tances

Engel Let s say that tendon is a relatively den e tissue I think it would probably have around 0 1 equivalent of colloid in it If you project a line through that point to the blood level then total calcium should be somewhere around 0 01 equivalent [*Additional comment by Dr Engel subsequent to the Conference* Subsequent experiment have shown thi estimate to be in error and much too high Rabbit gastrocnemiu tendon

showed r values of 0 01 equivalents of colloid per liter which would give calcium levels close to that of blood]

Howard Per what?

Engel Per liter

Bartter Ten milliequivalents per liter

Harrison Of water?

Engel Of total tissue[13]

Handler That is a lot of material It is five times the concentration in plasma

Engel But would you not expect it to be high if for no other reason than the Donnan effect and the binding of calcium by the colloid?

Handler There should be at least as much calcium in the free fluid as one can predict from the Donnan effect That much can be determined What I did not understand and the reason for my earlier question is the concentration as shown I thought you said you had worked at values of approximately 0 01 molar but your nomogram runs through an area from 0 001 up to 5

Engel The 0 001 mols refers to blood

Handler Yes but the concentrations given for blood here are the same as those presumably They are of the order of magnitude if not identical with those expected in the free interstitial fluid did they correspond to the medium in which you made your measurements?

Engel We used a rather un physiologic concentration of calcium

Handler What I do not understand is how you can construct this nomogram at these values when you made your experiments at different levels Did you perform your experiments at these levels as well?

Engel Yes we did We also used 0 005 molar calcium with similar results The nomogram is calculated from the equilibrium constant K

Armstrong Gentlemen I suggest if there is no objection that we now consider other aspects of the transport of mineral ions Nothing as yet has come up about absorption and excretion of calcium I understand that Dr Neuman has some data on a biological proces having to do with clearance studies in the kidneys

---

[13] Since the d r ... nnectn ... ut one the results might be expressed in eq ... n of

Ha dler May I terject a quest on Dr Armstrong? I do not qu te see that these stud es contrad ct tl e hypotl e s to wh ch you referred earl er h cl l as l cen developed at tl ese Conferences to descr be the cond t ons when calc ficat o shall or shall not occ r

Ir stro g Well t may be that m que t on v as put because of my o vn lack of kno le lge about exa tly v l at Dr Engel has descr bed

Ha dler If one th nks of col o d the m copolysacel ar des chon drot n sulfa e an l so fo th exchange res ns w th an affin ty for calc u n l t Dr E gel has done to quan tate the s t at on and the state ho v m cl of such calc m b nd g one may expect at a g en level of calc um in tl e flu d p l a e Tl s s n no sense tl e rej ct on of that hypothe

Ir s ro j M troul le arose from the not on that connect t ss es l cl do not calc f also ac umulate calc um

Ha dler There s another po nt to th t hypotl es s f you b nd calc um nto chond o t n lf n e o m t auton a call get apat e format on

Ir stro g W ll tl e d fferen e p obabl l e n the quant tat a pects of m eral l d n or througl factors wl ch n ay operate to alter for ex l p ospl ate o co cen rat on n tl e t sue What ould ou say as to the relat e order of n gn t le of calc um b nd ng as o t ha e measured t l et een art lage a d l n

E g l Well I th nk t s a atter of sat rat on I th nk tl at tl e prote n s al t half as at rated n sk n (5 per ent) as t s cart lage (8 per cent)

Follis You see n hat Dr Engel as measur ng he would l a e to n ea e non cal f e l cart lage an a

Copp Is tl ere a v d fference bet veen ord nary cart lage and the on calc e l cart lage fo l n tl e rncl t c ep pl seal reg on?

E g l I cannot ans er your quest o d rectl l ut I can tell ou th s —and tl s s ant pat ng ometl ng tl at I ant to say later— f you g e p ratl ro l extract to an n als a very sl ort t me you decrease the de t of c ll lal cl arge b abo t o e tl rd I mag ne tl at m a rach t c a n l electr etr cally ou o ld get the same sort of re lt Tl e den s t of negat el cl arge l collo l o ll l e le s v l l s to say tl en tl at t calc l nd g capac t o ll be decrea ed

H n ll r Ho long does that take?

E el Ol t takes place th n 12 l o rs It m ght occ r th n a couple of l o rs We l ave not mea ured it earl er tl an abo t 12 l ours

Foll s That s n sk n?

Engel In the epiphysis In the skin too the ability to bind calcium is reduced by giving parathyroid extract in other word all of these connective tissues are affected

Armstrong I think you had better give us that work now I will ask Dr Neuman to wait Would you mind describing to us your other studies?

Engel I do not want to monopolize this discussion

Armstrong I do not think you are

THE EFFECT OF PARATHYROID EXTRACT ON GROUND SUBSTANCE AND CALCIUM OF BONE[1]

MILTON B ENGEL HUBERT R CATCHPOLE and NORMAN R JOSEPH

Fro tl e Depa t ts of De al Tl rap t a d Ortl odo t a of Pa l ology a d of Cl e str Tl e Colleg s of Dc t str Med c e a d Pla a y Un v rs t of Ill o s Cl ago Ill o s

Ir s ro g Please l eg n Dr E gel

F gel I would l ke to make a atenent l cl o ta s son e general ews al t tl e a re an l l el a or of tl e gro l sub tan e of b n From l th electrocl en cal an l b to l en al e de ce tl e j ropert es of tl e n atr x re en bl tl o e of a l gl ly a gg egated egat el cl arged collo d Cl co j r te ns c st tute a er n po tant omponent of tl s gro nd sub sta ce As tl other conne t e t ue tl e teract on th o s related to tl e state of tl e c llo d and th re j e of l o es to son e l orn one tl e gro l t l ance n a l e o e dej l er z l an l tl e l nd g of cal c u tl s affecte l Tl aj parentl oc rs the often g an l the s lut on of l o e tl at follows the a ln n trat on f paratl ro d extract We bel e e tl at tl e e at o of tl e erun n coj t Te el and tl e rea ed excre t o of ur nary uc j rote tl e r e f llo ng nject on of j aratl ro d extra t act all rej re e t a lencl n t f s l l le re l es of tl grou d s b tance fr n tl e affected b e

[1] T h va su p ed l g an f m he A n can Ca er Soc ety re om m d l b th C mm ee on C o of Na al P a c Coun l f on he C adua e S h l U e y f l an l f m he M d l Pe ea d De e op men l a d Off e of e S g C ne l D partm t of e Army Co a t No DA 4 007 MD1

e h I a l Ca pcl H P The Orga za of C nd Su tance and l a n n Memb an a d l s S g fca e n I ue I u y D ea e and C row l l J l 8 457 (194

Cobb J TA M p a D f Cl o n a l Gly p o s Cr r a l J z a Ma a r f l a r g Form M S Thes Un ers of Ill n (1948

I gel M B T e Mot l zat f Mucoj h I a r l F x act l l Pa 3 339 (1)

H ll M G l S l ra P S l a l C a f r B e F na a l l s ru n I l D The Un ers y of Cl a (1 51)

J s N R F ge M B a d Ca l pc H F In e a f I a d C n ec e T ue B c Bo a l 8 S S (1

Fig 40 Sections of Bone and of Kidney Tubule Showing the Effect of Large Amounts of Parathyroid Extract

All of the tissues were fixed by freezing-drying and stained for carbohydrate containing substances with periodic acid leucofuchsin reagent after ethanol denaturation. The animal was given three injections of parathyroid extract totaling 1 150 units in 72 hours and killed 24 hours after the last injection.

→

It po s ble to sual ze the ground ubsta ce or the gl coprote n com po ent of t u sect ons of un lecalc fied bone fxe l b freez ng d ng and sta ned tl tl e p od c ac d leucofu l n re ge t Young bo sp cules ll sta j nk to re l th tl s reagent Tl e st nab l ty of older sp c les red ced to onl a fa t rea t o It has bee assun ed a d Cobb as one of the f rst to suggest t n a sfec fic a pl cat on of the h potl es of Gersl and Catchpol tl at th fa lure to sta n reflected a n ask g of re act e gro ps n l one such m ght occur f tl e bone collo d ere ery l gl l aggre a ed O the other hand f you nje t parath ro d extra t ratl er la ge doses as e l a e done tl the rats and rabb ts do es of 500 to 1000 u ts o er a 24 l our per od of t me the organ c matr x of tl e l o e dergoes chan es and sp cules vh ch formerl ou d sta n onl ver l gl l v tl tl e per od c ac d le cofucl s reagent o ta n q te ntensely

F gu e 40 A sl o a l one sp cule fron an an mal tl at as g n s cl a lo e of paratl yro d extract Yo can see the fibr llar tr cture of tl e con t g ous connect e t sue It appears that tl s t s e as formerly a part of tl e bo e an l a large part of tl e gro n l s bstance has d ol ed nn a k n t lea ng ts f br llar charac er appa e t It s ver s m lar to tl e l o o n crograj l sho n by Dr Pob nso a certa ense The d n egrat on of a l o e sp c le shown n F gure 40B

McCa e Yo are g ng an e or ou lo e f ara l ro l ext act f r b o l pl olog al l m ts

E gel Yes

M Ca e Ha e contro le l tl at n any va b g nge ormou doses of so e n lar sol t on I n ean a solut on conta n ng n lar amo nts of ro e

E gel Ye bo e gan a globul n and al o large lo es of sal ne ne l er of wl cl l a e tl at effect

A pa t of he p a y d ex a t wa supp ed ough e k nd e of E L y a l Co Ind nap

---

CT O OF I f The bone s and the conn e ue a e deep y a ned S me of the fibe s appea to be con nuou w h he depo m ed bo e m x Ma fi 650 X B—T e large da k a n ng aggrega e a e d n eg at ng p ece of bone sp cu es Magn fi a o 650X

F T O S K D EY TUB LE C—La ge ac l ua g o o g anu es a e p es n n a c o s sec ion of a p ox mal c n o u ed ubu e Magn ca on 1 200 X D T p epara on has be n t ea ed l mon a e n ganc p o pha e-ca bona The g p en ca t n he d la ed k dney ubu on a n pl e-ca bona e de p Magn fi a n 650 X

[Pe luced b perm f om Eng l M B Tl e Mob z n of Mu p o e n b Para l I a I h Pa h 53 33 (1 52)]

The Depolymerizing Effect of Parathyroid Extract on the Connective Tissue of Bone

If you examine the bone and cartilage of these animals carefully it is clear that the architecture has been changed Ice crystal artefacts (which are *artefacts of freezing drying*) are very prominent indicating that there has been increased water up-take When these animals are studied electrochemically as I have indicated previously the results show a reduced density *of negatively charged colloid* This is to say that the ability of the matrix to bind calcium is also considerably reduced Analysis of the bones (including the marrow) for water soluble alcohol insoluble mucoproteins showed *these substances to be increased* following the administration of parathyroid extract These changes in stainability reduced density of negatively charged colloid and the increase in water soluble mucoproteins are criteria which have been regarded as characteristic of depolymerized connective tissue [134 140]

Accompanying these changes in the extracellular physicochemical phases are changes in the connective tissue cells and these have been described particularly carefully by Heller McLean and Bloom [141] The cells may undergo transformations involving the osteoblasts osteocytes osteoclasts and reticular cells The amount of intracellular glycogen in osteoblasts and osteocytes is reduced The macrophages contain phosphate carbonate in association with aggregates of glycoprotein Presumably these are phagocytized residues of bone Some of the cells of the bone and contiguous connective tissue appear to be necrotic

We have no information about the *intermediate steps of this hormonal* effect but these are two tentative explanations which we are currently investigating (1) The hormone stimulates the mesenchymal cells to produce depolymerizing enzymes *forming soluble fractions of bone and cartilage or* (2) the hormone alters some other metabolic activity of the cells producing intermediate substances which dissolve bone and cartilage

I might mention at this point that electrochemical results show the density of charged colloid of other connective tissue such as skin gingiva and dentin also to be decreased as a result of the hormone effect This interpretation also is supported by histochemical studies [142]

---

[140] Joseph N R Engel M B and Catchpole H R Homeostasis in Connective Tissue to be published

[141] Heller M McLean F C and Bloom W Cellular Transformations in Mammalian Bones Induced by Parathyroid Extract *Am J Anat* 87 315 (1950)

[142] *Bloomfield J R and Hayes K* An Effect of Parathyroid Extract on the Ground Substances of Skin *Bull Alumni Assoc School of Medicine U of Chicago* 8 4 (1952)

The Nature of the Mucoproteins in Blood and Connective Tissue

I want to make a few comments about the mucoproteins specifically. The mucoproteins as components of both blood and connective tissue would be expected to be involved in the equilibrium between the two. Gersh and Catchpole have suggested that the increase of the serum mucoprotein level which had been observed by Siebert, Winzler and associates[14] Shetlar and associates[145] and a number of other people in a variety of disease states was related to some change that was occurring in the connective tissue ground substance. Catchpole[6] subsequently was able to demonstrate that in mice bearing transplantable tumors the connective tissue about the tumors contained more water soluble glycoprotein and the blood levels of mucoprotein in these animals was elevated. A similar change was observed in scurvy by Pirani and Catchpole[147] We thought that if the bone ground substance is being dissolved and if this mucoprotein is an important part of it the blood mucoprotein might rise following administration of the hormone. This was tested in adult rats who received doses varying from 10 to 1600 units over periods up to 96 hours (Table XV)

TABLE XV

The Effect of Parathyroid Extract on the Serum Mucoprotein Level of the Rat

Dose	Duration of Experiment	Serum Mucoprotein as Carbohydrate
(units)	*(hours)*	*(mg /100 cc)*
Control	—	16
10 to 30	19	23 25
100 to 300	24	28 40
800 to 1600	24 96	35 73

Seibert F B Seibert M V Atno A J and Campbell H W Variation in Protein and Polysaccharide Content of Sera in the Chronic Diseases Tuberculosis Sarcoidosis and Carcinoma, *J Clin Investigation* 26 90 (1947)

Winzler R J and Smyth I M Studies on the Mucoproteins of Human Plasma II Plasma Mucoprotein Levels in Cancer Patients *J Clin Investigation* 27 617 (1948)

Shetlar M R Foster J V Kelly K H Shetlar C L Bryan R S and Everett M R Serum Polysaccharide Level in Malignancy and in Other Pathological Conditions *Cancer Res* 9 515 (1949)

[6]Catchpole H R Serum and Tissue Glycoproteins in Mice Bearing Transplantable Tumors *J Soc Exper Biol and Med* 75 221 (1950)

Pirani C L and Catchpole H R Serum Glycoproteins in Experimental Scurvy *Arch Path* 51 597 (1951)

In control animals the serum mucoprotein level expressed as carbohydrate was around 16 mg[149] In animals treated with massive doses levels as high as 4½ times this value were recorded One group of adult rats was given 300 units and then sacrificed at varying intervals Control values for serum mucoprotein were exceeded at 3 to 5 hours The maximum elevation was observed at around 30 hours and at 48 hours the level was still abnormally high (Figure 41) We found that the kidneys showed severe damage when the protein levels were very high

Howard What happened to your calcium coincident with the rise?

Engel I do not know I would like to know but we did not make that determination

The Relation of Mucoproteins to Kidney Damage

The kidney tubules were plugged with a material which contained carbohydrate and which has been shown by ultraviolet light absorption to contain protein also It is interesting that the tubule cells themselves showed large aggregates of glycoprotein granules under the influence of this hormone Perhaps this represents the precursor of the tubular casts (Figure 40C and 40D)

These casts sometimes contain phosphate deposits which are presumably calcium phosphate One might anticipate here again that because of the Donnan effect this mucoprotein would tend to increase the calcium ion concentration leading to renal calcification Perhaps in other instances where renal stones are formed elevated levels of serum mucoproteins together with increased renal elimination of these substances could be precipitating factors Such a series of events might occur in conditions where there is extensive bone resorption or considerable loosening of the connective tissue

The Excretion of Mucoprotein in Urine

It occurred to us that if this material were demonstrable in the kidney it might also occur in the urine Recently Tamm and Horsfall[150] had shown

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[1] [9]Serum mucoproteins were estimated by the method of Winzler and associates[14]

[149]Winzler R J Devor A W Mehl J W and Smyth I M Studies on the Mucoprotein of Human Plasma Determination and Isolation *J Clin Investigation* **27** 609-616 (1948)

[150]Tamm I and Horsfall F L Jr Characterization and Separation of an Inhibitor of Viral Hemagglutination Present in Urine *Proc Soc Exper Biol and Med* **74** 108 (1950)

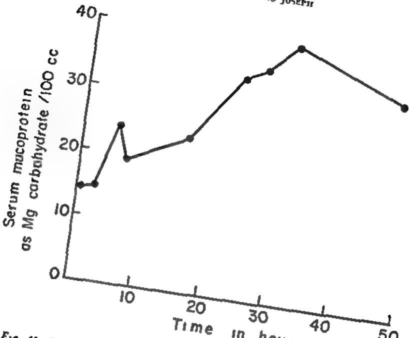

Fig. 41. The Effect of Parathyroid Extract on the Serum Mucoprotein Level of Rat.

The animals were given 300 units of parathyroid extract.

[Reproduced by permission from Engel, M. B.: The Mobilization of Mucoprotein by Parathyroid Extract. *Arch. Path.* 53:339 (1952).]

that human urine contains mucoprotein which inhibits virus hemagglutination. Using their method, we precipitated out the urinary mucoprotein of the rat.

The mucoprotein* excretion in adult rats was studied over a 3-day control period and compared with that observed over a similar period following the injection of 1,000 units of parathyroid extract. Approximately 2½ times as much mucoprotein carbohydrate was excreted during the post-injection period as before (Table XVI).

---

*The urinary mucoprotein was precipitated with 0.5M NaCl and determined as carbohydrate using a mannose-galactose standard.

TABLE XVI

The Excretion of Urinary Mucoprotein by Rats following the Injection of 1000 Units of Parathyroid Extract

Period of Study	Urinary Mucoprotein as Carbohydrate	
	Mean	Range
	(mg)	(mg)
3 day control period	1 06	0 35 1 92
3 day post injection period	2 79	0 52 5 15

Summary The Effect of Parathyroid Extract on the Ground Substance Constituents of Bone

Summarizing then we may say that a study of the effect of parathyroid extract on bone by various methods has indicated responses which we feel to be similar in certain respects to the action of several other hormones on connective tissue There occurs primarily a disaggregation of the glycoprotein ground substance with the production of soluble carbohydrate fractions This is shown histochemically and by analytical studies and is inferred from the increases in the blood and urinary mucoprotein levels

Elsewhere [0] we have discussed the effects of certain hormones on connective tissues in terms of a two phase equilibrium between the soluble and the insoluble colloids The relationship of this equilibrium to the distribution of calcium and sodium ions was expressed in terms of the nomogram[152] to which we referred previously (Figure 39) Under homeostatic conditions the physical state of the colloidal bone matrix may be represented then by the line at the upper limit of the nomogram but since the administration of parathyroid extract causes an elevation of the blood calcium level the effects of this hormone may involve non homeostatic displacements of equilibrium in all parts of the connective tissue continuum

Conference Discussion

Follis Is there any reason to think that this is specific for parathyroid hormone or does it result from any destructive process in bones?

Engel I think any destructive process of the bone or in connective tissue generally would lead to these very same results

[152]Engel M B Joseph N R and Catchpole H R Equilibrium of Calcium and Other Ions in Connective Tissue *TRANS MACY CONFERENCE ON METABOLIC INTERRELATIONS* 5 000 (1953)

Robinson Maybe you would not want to answer this but would there be any relation between the hydration of the mucoprotein or the ground substance of these connective tissues and their state of polymerization?

Engel I believe there would be. I believe that the more highly hydrated they are the looser they are and the less hydrated they are the tighter they are. This is just another aspect of the same phenomenon.

Howard Dr Engel, may I ask you a couple of questions?—because what you have said appeals to our theoretical concepts about the breaking down of matrix and releasing the matrix, the lime salts and everything at the same time. Some years ago we tried to find a mucopolysaccharide in the blood of people with hypercalcemia looking for something that would carry the extra calcium in the blood because at that time we thought it was already supersaturated. Dr Bacon Chow did an electrophoretic pattern for us on the blood of hypercalcemic people after their hypercalcemia had been cured—(the patients consisted of people with parathyroid tumors before and after their removal, people with sarcoidosis who had high calciums but without protein abnormalities that we could tell electrophoretically or by any other methods)—and we could find none of Winzler's mucopolysaccharide at a pH of 4 electrophoretically and we could not get any increased polysaccharides in the blood of these people with parathyroid tumors as compared before and after the hypercalcemia had disappeared. I wonder if you could explain that.

Then another thing that we did was that Dr Rubin got some chondroitin sulfuric acid reasonably pure—I will not say that it was purified—and we put that against calcium solutions in the ultrafilter and found that, as expected, it bound enormous quantities of calcium. But when we added some to normal serum and put it in the ultrafilter it completely lost that power to bind; in other words, something within the serum negated it completely and it had no effectiveness whatever to carry more calcium so that the ultrafiltrate was exactly the same as before we added the chondroitin sulfuric acid to the system.

Lastly, the material that Winzler commented on rises in coronary occlusion, cases to quite high levels, as well as in lobar pneumonia and in all sorts of other disease states in which I have never seen anybody with hypercalcemia or any evidence of active bone destruction over and above the catabolic reaction that you might expect to go with it. I was wondering if you could put those three negative appearing facts into what you have just told us.

Engel About the rise in pneumonia, it has been suggested that in a number of nonspecific disease states the connective tissue is being disaggregated as, for example, in the lungs in pneumonia. As a result more

TABLE XVI

The Excretion of Urinary Mucoprotein by Rats following the Injection of 1000 Units of Parathyroid Extract

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15 Engel M B Joseph N R and Catchpole H P Equilibrium of Calcium and Other Ions in Connective Tissue *TRANS MACY CONFERENCE ON METABOLIC INTERRELATIONS* 5 000 (1953)

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Engel About the rise in pneumonia it has been suggested that in a number of nonspecific disease states the connective tissue is being disaggregated as for example in the lungs in pneumonia As a result more

soluble fractions of ground substance are getting into the blood where they are reflected as an elevation of the mucoprotein level The serum mucoprotein level can be elevated in scurvy also

As to the failure of chrondroitin sulfate to bind calcium in the presence of serum I do not know the answer Perhaps the chondroitin sulfate is combining with some protein of the serum which competes with calcium The material that one deals with in bone may be somewhat different from the degraded type of chondroitin sulfate that one would have—

Follis But you do not know whether or not you are measuring chondroitin sulfate do you?

Engel Where in the blood?

Follis Yes

Engel You know that you are measuring a protein which has been characterized by Winzler I think there are two peaks demonstrable by electrophoresis It is not too heterogeneous a material About not being able to demonstrate material electrophoretically I have no explanation

Howard I can not get very excited about a small coronary occlusion giving you enough outpouring from that area The lung I can not argue about but I do not see how a little coronary occlusion could give you enough ground substance in the circulation—

Engel Well Dr Fremont Smith encouraged speculation so I will take advantage of that opportunity Some of us think that some coronary occlusions could be caused by elevated serum mucoprotein levels Perhaps the serum mucoprotein combines in someway with material of the vessel walls so it is a question of what comes first

Howard But it comes right down again in two or three weeks after coronary healing

Engel Maybe the initial disease state has changed too

Armstrong Of course you get other effects you get leukocytosis in coronary occlusion for example In other tissues that are affected by disease mucoproteins might be disaggregated

Engel People are very sick with coronary occlusions which means that there is certainly a non homeostatic state

Rubin I would like to suggest the possibility of the implication of magnesium in some of these situations We have been studying and perhaps we will have a chance to talk about the relationship of calcium and magnesium particularly their ratio and it turns out that in cardiac conditions and in pneumonia there is a shift in the magnesium calcium ratio

I was wondering whether the two are not related to your binding by muco protein in the serum In hypoxia and anoxia there is postulated a change in permeability of the membrane which would permit rapid shifts in magnesium which ordinarily you might miss if you analyzed the magnesium directly but when you begin to consider both calcium and magnesium simultaneously and examine the ratio of the two the changes become a little bit clearer

Engel Preliminary experiments show that this material will bind magnesium

Copp Mr Chairman I would like to compliment Dr Engel on this very stimulating concept of the nature of the ground substance and I hope that perhaps he may be able through this to explain two problems in connection with ground substance of bone The first is the difference in the nature of cartilage which does not calcify e g articular cartilage as compared to that in the region of epiphyseal growth where you do get calcification The second problem concern the very specific uptake by the osteoid matrix including the uncalcified osteoid matrix of the rachitic animal of heavy metals such as yttrium plutonium cerium and so forth We also found that the uptake of plutonium was unaffected by giving parathormone or by other procedures which attack the bone There may be some explanation on the basis of this colloidal reaction for there is something very specific in the ground substance of calcifiable matrix which makes it react with these heavy metals

Engel I do not know too much about it but I know that last year Dr Neuman reported on th binding of calcium by chondroitin sulfate and perhaps that is the thing that is specifically implicated here As far as calcification goes as far as the apatite formation is concerned you may have a difference in the metabolism of phosphate in the articular areas as compared with the epiphysis

Copp You can follow the bone down from the articular cartilage which is not calcified and you run directly into a region where active calcification has taken place Both are equally accessible to the blood stream the cells look very similar but there is a sudden change in the reaction

We also observed in connection with plutonium uptake that when we gave young rats up to 1000 units of parathormone and combined this treatment with administration of zirconium citrate there still was no significant effect on uptake or removal I do not think this disproves a change in the colloidal nature but it does indicate that there is little noticeable effect on the specific combination of matrix with these heavy metals

THE RENAL CLEARANCE OF CALCIUM IN NORMAL DOGS[133]

WILLIAM F. NEUMAN and PHILIP S. CHEN, JR.

From the Atomic Energy Project, The School of Medicine and Dentistry, University of Rochester, Rochester, New York

Armstrong: We have an hour now for excretion, and I am going to ask Dr. Neuman to open the discussion of this topic.

Neuman: I am going to state our work very briefly. I am giving these data knowing full well that they may mean very little because we are entirely dependent on guesses as to the state of calcium in the blood. I think that in this particular case the methods employed are extremely important. The data cannot be generalized.

There is very little in the literature on the renal excretion of calcium. Probably the primary reason is the difficulty in obtaining sufficiently large blood samples to determine both the total and the ultrafilterable calcium and to do it with precision. It was the development of a rather simple apparatus for preparing ultrafiltrates that led us to attempt the study of calcium clearance by the kidney.

Procedures Employed in Current Study

RENAL CLEARANCE

The experiments were performed on three trained, unanesthetized female dogs after an 18-hour fast, during which water was allowed *ad libitum*. The average weight of the animals was about 8.3 kilograms. Clearance periods were run while the dog was loosely restrained on an animal board in the supine position. Urine samples were obtained through an indwelling rubber catheter. Clearance periods were 10 to 30 minutes in length. Each collection period was terminated by at least two rinses with distilled water followed by an air wash, except during rapid urine flows, when only an air wash was used. Blood samples were withdrawn at the mid-point of a clearance period through a jugular vein and centrifuged immediately to obtain serum. Intravenous infusions were effected by inserting a soft poly-

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[133]This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, New York.

ethylene catheter through one external jugular vein and drawing blood samples through the other vein

Calcium concentrations in blood serum serum ultrafiltrate and urine were determined by flame photometry using the Weichselbaum Varney flame spectrophotometer Sodium and potassium concentrations in the urine were determined using the same instrument Inorganic phosphate levels were determined by the method of Fiske and Subbarow [5] Inulin was used to estimate the glomerular filtration rate in some of the early experiments and when this was done it was administered subcutaneously in a dose of 25 cc of a 20 per cent solution in 0 6 per cent saline about an hour before commencing the first clearance period Inulin levels were determined by the method of Roe Epstein and Goldstein Subsequently endogenous creatinine as determined by the method of Hare was used for measuring the glomerular filtration rate and in our hands the clearances of these two substances (inulin and creatinine) were essentially equal

THE ULTRAFILTRATION APPARATUS AND ITS OPERATION

Figure 42 shows the ultrafiltration apparatus It is a sintered glass filter which has been closed at the bottom and a side arm attached The small sample of serum is placed in a cellophane bag and the bag is made to form a loop which is inserted in the filter The gas mixture (95 per cent O_2 5 per cent CO_2) is passed through until equilibrium is reached and by attaching the rubber tube the whole ultrafiltrate and the sample that is being ultrafiltrated are in the same gas mixture As fluid passes into the chamber the pressures are equalized This apparatus is centrifuged at various rates of speed With this equipment and the use of the flame photometer we can obtain values for the total calcium the sodium the potassium the phosphate and the ultrafilterable calcium from an analysis of about 10 cc of blood

Butler Blood or plasma?

Neuman Serum of course is what we have analyzed However the volume of whole blood taken at any one clearance point is only 10 cc

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Fiske C H and Subbarow Y The Colorimetric Determination of Phosphorus *J Biol Ch* 66 375 400 (1925)

Roe J H Epstein J H and Goldstein N P A Photometric Method for the Determination of Inulin in Plasma and Urine *J Biol Chem* 178 839 845 (1949)

Hare R S Endogenous Creatinine in Serum and Urine *Proc Soc Exp Biol and Med* 74 148 151 (1950)

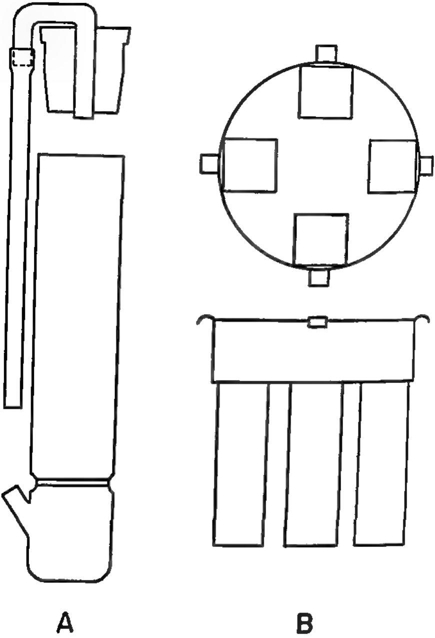

Fig 42 Schematic Drawing of Ultrafiltration Apparatus

A—Filtration apparatus made from a straight sealing tube 25 mm in diameter with a 20 mm fritted disc *B*—Top and side views of the centering device

TABLE XVII

The Ultrafiltration of Calcium Solutions
(Ca Solutions of 10 to 20 mg per 100 cc)

Solution	Revolutions	Ultrafilterable	
		Calcium Compound Only	Calcium Compound in 0 15 M NaCl
	(per minute)	(%)	(%)
Calcium chloride	2000	97	98
Calcium gluconate	2000	96	98
Calcium Versenate	2000	86	100
Calcium citrate	1500	89	—
Calcium citrate	2000	—	95
Calcium citrate	2500	84	—

In Table XVII are collected a few data relating to the operation of the ultrafiltration apparatus I should preface my remarks by citing three facts about the particular type of membrane employed 1) it does not pass detectable amounts of plasma protein 2) it does not pass detectable quantities of hydrocolloid (beryllium hydroxide) and 3) it is permeable to inulin Table XVII shows that in the presence of approximately physiological concentrations of sodium chloride diffusible calcium (whether present as a free ion or as a complex) was nearly 100 per cent filterable In the absence of saline there was some tendency for the larger ions to resist ultrafiltration

Gutman How long do you centrifuge?

Neuman About three hours

Sobel Are these pure solutions?

Neuman Yes these are solutions which have been synthetically prepared from C P reagents

Table XVIII presents the data on the effect of the speed of centrifugation on the ultrafilterable fraction Serum was employed in these studies and while there may be a slight tendency for the ultrafilterable fraction to decrease at high rates of centrifugation the changes observed are not significant

Armstrong Did you say what kind of membrane you employed?

Neuman Cellophane

TABLE XVIII

The Effect of Centrifugation Speed on the Ultrafilterable Calcium in Dog Serum

Revolutions	Ultrafilterable Calcium
(per minute)	(mg per 100 cc)
1500	6 1
2000	6 1 6 2
2250	6 3
2500	5 9 5 9

Total calcium = 13 3 mg per 100 cc 3 hour centrifugation

Harrison Is this cellophane commercially available?

Newman Yes this cellophane is sold commercially under the trade name *Visking tubing*

I have summarized the technique employed in the determination of the ultrafilterable fraction of serum calcium In the subsequent work two assumptions have been made (1) It is assumed that this technique reproduces the situation occurring in the renal glomerulus that we may calculate the calcium filtered by the glomerulus from the average glomerular filtration rate as determined by inulin or creatinine clearance and the level of ultrafilterable calcium as measured by our technique *in vitro* (2) This assumption is based on an early publication[137] which claims that nearly all ultrafilterable calcium is in the free *ionic* state As will be shown later this is a most important factor in determining the calcium clearance

Results Obtained in Normal Dogs

EVIDENCE FOR ACTIVE RESORPTION OF FILTERED CALCIUM

In Table XIX are compiled the results of a number of studies in normal dogs It is clear even from these data that calcium is actively reabsorbed because on the average 99 2 per cent of the filtered calcium was reabsorbed The average calcium clearance was 0 37 cc per minute I think it is important to re emphasize the magnitude of the clearance ratio The highest

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137 McLean, F C and Hastings A B A Biological Method for the Estimation of Calcium Ion Concentration *J Biol Chem* 107 337 350 (1934) The State of Calcium in the Fluids of the Body I The Conditions Affecting the Ionization of Calcium *J Biol Chem* 108 285 322 (1935)

TABLE XIX

The Calcium Clearance of the Normal Dog

	Average	Range
	(cc per min)	(cc per min)
Urine flow	—	0.01–4.1
Glomerular filtration rate	37.7	30.5–45.6
Calcium clearance	0.37	0.14–0.61
	(mg per 100 cc)	(mg per 100 cc)
Total serum calcium	11.9	11.3–12.8
Ultrafilterable calcium	5.8	5.0–7.0
Ratio $\frac{\text{Calcium clearance}}{\text{Glomerular filtration rate}}$	0.008	0.004–0.016
	(mg per min)	(mg per min)
Calcium filtered	2.14	1.75–2.74
Calcium excreted	0.017	0.007–0.032
Calcium reabsorbed	—	1.74–2.71

TABLE XX

The Effect of Diodrast and Para aminohippuric Acid (PAH) on Calcium Excretion

Drug	Dose	Period	Glomerular Filtration Rate	Calcium Excreted	Clearance Ratio
			(cc per min)	(mg per min)	
Diodrast	40 cc of 35% Soln	Control	34.8	0.04	0.024
			35.8	0.04	0.023
		Experimental	26.8	0.16	0.107
			28.1	0.09	0.052
			29.0	0.09	0.058
PAH	50 cc of 10% Soln Prime then 2 cc/min	Control	35.5	0.01	0.005
		Experimental	22.0	0.43	0.31
			20.8	0.36	0.28
			16.1	0.34	0.33

excretion observed in the normal dogs amounted to only 1 6 per cent of the filtered calcium

THE EFFECTS OF DIODRAST AND PARA AMINOHIPPURIC ACID

Since the data indicated the occurrence of an active process it was of interest to study the effect of the administration of diodrast and of para aminohippuric (PAH) acid on the calcium clearance These data are assembled in Table XX Both agents increased the excretion of calcium but of the two PAH was by far the more effective causing a 60 fold increase in the clearance ratio Even in this case however with maximum inhibition 70 per cent of the calcium filtered was reabsorbed

THE EFFECT OF CALCIUM ADMINISTERED INTRAVENOUSLY

In Figure 43 are presented the results obtained following a single intravenous administration of calcium gluconate It was of interest here that the injected calcium seemed to disappear It did not appear in the blood and it did not appear in the urine We have assumed that the skeleton had soaked up 80 per cent of the injected dose

Howard How much did you inject?

Neuman 60 to 70 mg

THE DELAY IN CALCIUM EXCRETION

Another important phenomenon to be noted is the pronounced lag in calcium excretion The urinary calcium excretion showed the most marked increase three to four hours after a single intravenous administration of calcium gluconate In subsequent experiments with calcium chloride, Figure 44 it was found that this lag period in the calcium excretion could be shortened by the preliminary administration of large amounts of water Even with hydration however the peak excretion was not observed until two hours after the intravenous injection

EFFECT OF ... PHOSPHATE

In Fi... ented the ... d during the ... of pho p... the total ... markedly and there was ... ormal tall ... le fraction The ph... co... ro... u this the renal ... d rise ... did, but to our

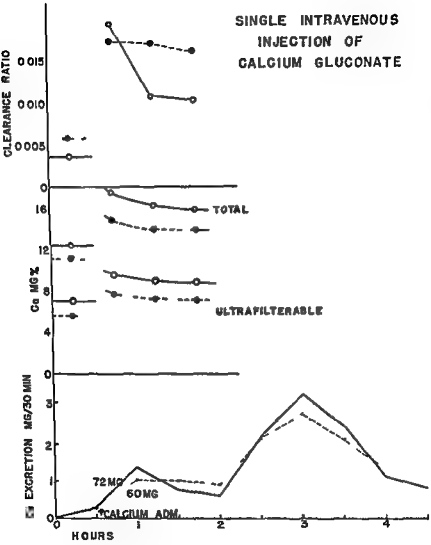


Fig 43 The Clearance of Calcium following the Intravenous Injection of a Single Dose of Calcium Gluconate

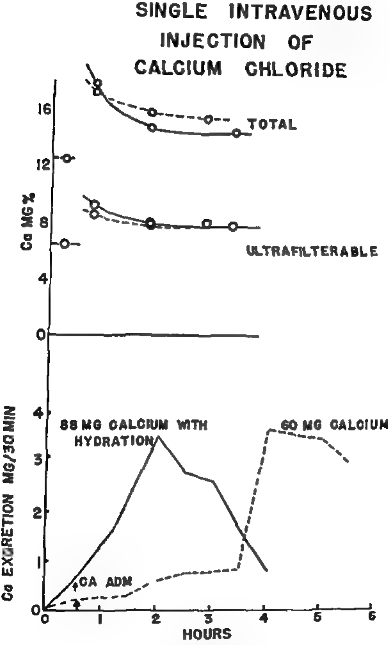


Fig 44 The Excretion of Calcium following the Intravenous Injecti[on] of a Single Dose of Calcium Chloride

Note the increased rapidity in calcium excretion following hydration

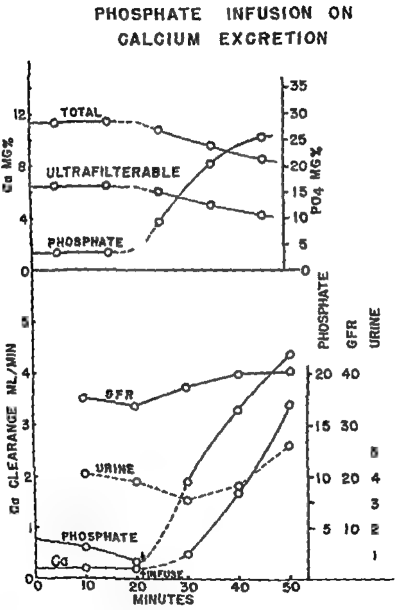


Fig 45 The Effect of Phosphate Infusion on Calcium Excretion

surprise so did the clearance of calcium We finally concluded that part of the difficulty might be attributed to the formation of a soluble highly associated complex of calcium phosphate This complex was described several years ago by Greenwald[158, 159] and in our own laboratory Dr Gosselin[160] has confirmed its existence

DEDUCTIONS CONCERNING THE STATE OF DISSOCIATION OF CALCIUM

From Gosselin s studies we were able to derive a rough dissociation constant from which it could be calculated that normally only a small fraction of the serum calcium could be bound in the form of a soluble phosphate complex

Sobel Do you mean a negatively charged complex?

Neuman Well I am not sure that it is negatively charged but it certainly is not positively charged Whatever the charge the complex ion is not adsorbed by a cation exchange resin

Shorr Do you still have those urines?

Neuman I doubt it very much

Armstrong If you have Dr Shorr will make some determinations for you

Shorr I would like to determine the citrate content

Neuman The citrate?

Shorr Yes Or perhaps you did?

Neuman No we did not

May I go on? Using the rough dissociation constant it was possible to show that only 20 per cent of the calcium in the excreted urine could be in the free ionic state It looks very much therefore as though the increased calcium clearance resulted from the reduction in the concentration of free calcium ion caused by the excessively high concentration of phosphate

Kramer Were there any calcium phosphate crystals in the urine?

---

[158]Greenwald I The Effect of Phosphate on the Solubility of Calcium Carbonate and of Bicarbonate on the Solubility of Calcium and Magnesium Phosphate *J Biol Chem* 161 697 704 (1945)

[159]Greenwald I, Redish J and Kibrick A C The Dissociation of Calcium and Magnesium Phosphate *J Biol Chem* 135 65 76 (1940)

[160]Gosselin R Unpublished data

Neuman I cannot answer that with any real assurance

Howard Did you do that experiment again with a lesser load of phosphate? You see you have put the levels into the colloidal range Twelve millimols per liter of phosphate is a tremendous amount of material

Robinson That is milligrams

Howard Is that milligrams?

Neuman The highest value is 25 mg per 100 cc

Howard Yes but that is very high

Neuman Yes it is very high

Howard Did you do any at about 10 or 12 mg per 100 cc? We did that in the human you see and got exactly the opposite result I wondered if it was the amount you gave that made the difference

Neuman I agree this represents a most un physiological condition

Partter Could not the effect of infused phosphate on calcium excretion be explained by a mechanism analogous to that which presumably applies to para aminohippurate? In each case there is an anion claiming excretion and calcium is merely one of the cations used to meet the demands of electrical neutrality

Neuman By all means your suggestion is logical The reason we are not employing such an interpretation is because of the data which are assembled in Figure 4C

THE EFFECT OF CALCIUM ADMINISTERED AS THE VERSENE COMPLEX

In this experiment calcium was administered in the form of the Versene complex In this case the calcium clearance was tremendously elevated At the highest level only 30 per cent of the filtered calcium was resorbed The excretion of calcium paralleled closely the excretion of the Versene

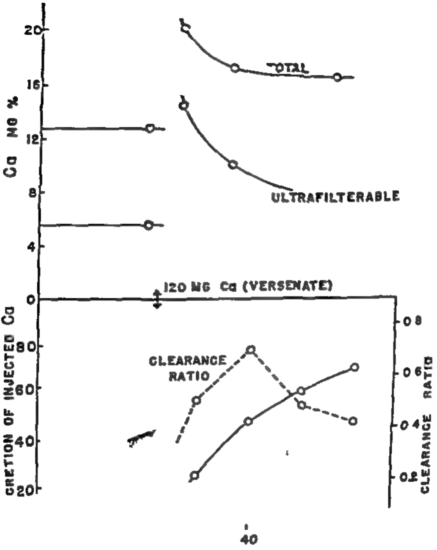
Shorr Did you add Versene or calcium Versenate?

Neuman Calcium Versenate was injected It is true that by this means we artificially raised the calcium level but the calcium is not free to be reabsorbed since it is in the form of an undissociated complex

Handler Can you explain the PAH effect on the excretion of calcium?

Neuman I do not think so The findings with PAH I believe are the result perhaps of non specific competition of tubular processes already working at maximum capacity because of an artificial diuresis We ob-

SINGLE INTRAVENOUS INJECTION OF CALCIUM-VERSENATE COMPLEX



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served that in addition to overloading the tubular capacity any material in the blood which will pass the glomerulus and form an undissociated complex of calcium also can increase the calcium clearance

Present Interpretation

We conclude that the clearance of calcium is determined by the ability of the tubules to function normally and by the ionic concentrations of calcium in the blood and in the tubular fluid

Conference Discussion

Kramer I suppose the calcium in these studies was determined before and after washing?

Neuman No Calcium was determined by the flame photometer The method is so sensitive that analyses can be performed directly on diluted serum

Shorr But you did not determine your calcium as calcium that was combined with the Versene?

Neuman No separation was made The Versene complex may be assumed to be 100 per cent undissociated Would you agree with this assumption Dr Rubin?

Rubin A hundred per cent covers a lot of ground Yes the degree of undissociation is very high

Neuman I agree Though even 99 per cent would not be a bad approximation

Rubin I would go higher than that—99 999 per cent

Neuman Well that is satisfactory for our purposes I believe therefore that the increase in the level of ultrafilterable calcium that is seen after the injection of calcium Versenate is due entirely to the material injected

Follis Your figures are lower than the ones which Dr Howard presented this morning

Neuman Somewhat Dr Howard s ultrafilterable fraction was approximately two thirds the total calcium Our fraction was more nearly one half

Harrison Have you determined any human ultrafilterable calcium by your technique?

Neuman Yes

Sobel Maybe the different pH control makes the difference

Neuman In our experiments pH is controlled by equilibration with oxygen CO mixtures

Sobel The pH in your studies is consistently 7.4?

Neuman Yes

Sobel And how about in yours Dr Howard?

Howard The pH values vary from 7.38 to 7.5 We cannot get them absolutely constant They vary from batch of serum to batch of serum

Neuman I did not mean to imply that we did not observe pH variation the pH of 7.4 is an average result

Armstrong I think Dr Howard also found that there seemed to be no dynamic equilibrium in the filtration

Howard I never could understand that I merely present all of these observations for you to explain

Neuman But your ultrafiltration is much slower than ours is it not?

Howard Oh yes it is much slower

Neuman How many hours does it take?

Howard Well 24 to 48 to 72

Neuman Are you not performing a dialysis?

Howard No because the contact is very abrupt You see the material rises right to the top of the mercury after it passes from the membrane so that the contact is immediately cut off from what has been filtered it goes right to the top of the mercury column What you obtain in the first two hours is the same as what you get in 24 hours it is the same as what you get at 72 hours I do not understand this Obviously the material that is held back is getting more and more concentrated as to protein and calcium and less and less as to water

Harrison Is there any possibility that the mercury could denature the protein?

Howard There is no protein passing the membrane I do not know

Harrison If the serum protein were denatured by prolonged contact with mercury its combining capacity for calcium could be changed

Sobel: There is another possibility that oxygen affects it. You used oxygen and CO didn't you instead of nitrogen and CO?

Neuman: Yes.

Howard: We did too. We used oxygen and CO.

Neuman: There appears to be a difference in the results obtained by the two techniques. Which method is more accurate cannot be decided with the present information.

Howard: What is the difference?

Neuman: The difference is in the percentage which is ultrafilterable as determined by the two techniques.

Harrison: Dr. Neuman gets about 40 per cent ultrafilterable calcium and Dr. Howard obtains about 70 per cent.

Neuman: Yes. That is the important difference.

Howard: Our studies were under much higher pressure. Dr. Neuman's studies were done under just gravity pressure and ours were under 200 mm. of mercury.

Follis: Dr. Howard had 8000 G.

Partier: Dr. Howard, did you say that the calcium concentration in the material left behind is going up?

Howard: In the ultrafiltrate it stays the same, so it has to be going up since there is less and less water in the material that is held back.

Storr: But it is lower in relation to the proteins, that is, the protein concentration is going up in relation to the calcium.

Armstrong: People who are familiar with what is understood about membranes—and I do not think it is very much—know that the quality of the membranes can be altered so that they are anion permeable or they are cation permeable. Although you both might have been using Visking, I am not at all sure that you both were actually dealing with the same membrane. Certainly, the nature of the membrane would have some importance, wouldn't it?

Howard: We soaked our membrane in distilled water before using it. We found that there was a tremendous difference if we did not do that. Did you do that, Dr. Neuman?

Neuman: Yes. One hour in distilled water. What was the difference you observed?

Howard I do not remember whether it was greater or less I think it was less

Neuman Actually the centrifugation is interrupted and the first few drops of ultrafiltrate are discarded This procedure minimizes errors due to contamination or dilution

Hodge I wish to make a comment bearing on this same filtration apparatus We began to wonder about how important the bicarbonate complexing of calcium might be and we studied this point using the same setup and using a cation exchange resin with Ca^4 (Neuman W F Hodge H C Morrow P E and Toribara T Y *U of Rochester Atomic Energy* Report UR—275 1953) We came out with a very clearcut answer Both of the stories checked perfectly or I should say reasonably The bicarbonate complex of calcium does occur to a minor extent and I think I can summarize the findings by saying that it certainly does not account for more than 10 per cent of the diffusible calcium so if any of you has wondered about it I think you can put the bicarbonate complex down as one that is not very important in calcium transport in the blood

Armstrong *How do you know that it is a bicarbonate complex* and not the CO affecting the complex?

Hodge The answer to that is that we did not prove that it was a bicarbonate complex but we did study the properties under varying pressures of CO

STUDIES ON THE PLASMA CLEARANCE VALUES OF CALCIUM

D HAROLD COPP

From the D partment of Physiology Faculty of Medicine University of British Columbia Vancouver British Columbia

Armstrong Dr Copp do you wish to make some comments?

Copp Mr Chairman I would like to present some data on another species the rat where the clearances were calculated by a much simpler and cruder method but where the findings in general confirm the results shown

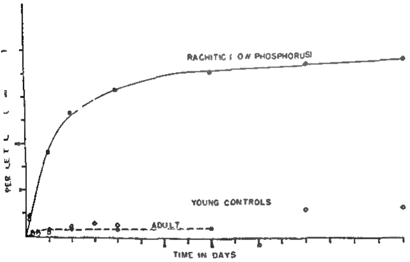

Fig 47 Cumulative Excretion of Radiocalcium in Urine (Expressed as Per Cent of the Administered Dose) following Intravenous Infusion

[Rep oduc 1 by permis ion f om C pp D H Ham lton J G Jones D C Thomp cn D M a d Cramer C Th Effect of Age and Low Phosphorus Rickets a Calcif cati n and the D tion of C tain Rad acti e Metals Bone *TRANS MACY CONFERENCE ON METABOLIC INTERRELATIONS* 3 243 (1951)]

In this particular experiment we plotted the cumulative excretion of Ca^{45} in the urine (Figure 47) and then determined the excretion rate by drawing slopes at various points on the curve. By dividing the excretion rate by the plasma concentration it was possible to calculate the plasma clearance values. These are shown in Table XXI, expressed as cc per minute per square meter of body surface. They are expressed on this basis so that values may be comparable despite the differences in size of animals. Adult rats and young rats have a relatively low clearance of either Ca^{45} or Sr^{89} with the tubules reabsorbing around 90 to 97 per cent, fairly close to the values which Dr. Neuman just reported for dogs. In our rachitic animals the clearance value was much higher. These animals were fed a diet low in phosphorus but with ample vitamin D. They developed low phosphorus rickets.

Handler: How was the rickets produced?

Copp: By feeding the animals a diet very low in phosphorus (0.034 per cent phosphorus), but otherwise adequate.[161] The diet contained adequate amounts of vitamin D (8 units per gram). The inulin clearance or filtration rate has been reported for the rat as 0.23 to 0.36 cc/min/100 sq cm.[18] Actually, there are not many reliable figures on filtration rate owing to the difficulty of measuring clearance by conventional methods in this small animal. Our results show that the normal clearance of calcium is very low but in low phosphorus rickets the clearance approaches the filtration rate. We also found that if we gave these low phosphorus animals sufficient

TABLE XXI

The Renal Clearance of Radiocalcium and Radiostrontium in Rats

State of Animal	Isotope Administered	
	Radiocalcium (Ca^{45})	Radiostrontium (Sr^{89})
	cc/min/sq m	cc/min/sq m
Adult	2.7	1.4
Young	0.6	1.6
Rachitic	12.0	13.0

Filtration rate = 23 cc/min/sq m

[161] Coleman, R. D., Becks, H., Kohl, F. V., and Copp, D. H.: Skeletal Changes in Severe Phosphorus Deficiency of the Rat. Tibia, Metacarpal Bone, Costochondral Junction, Caudal Vertebra. *Arch. Path.* 50: 209 (1950).

[18] Friedman, S. M., Mackenzie, K. R., and Friedman, C. L.: Renal Function in the Adrenalectomized Rat. *Endocrinol.* 43: 123-125 (1948).

phosphate to bring their blood phosphate value up to normal then the clearance dropped to a low level (0 6)

Sob l Without vitamin D?

Copp These animals did have vitamin D

Sobel I mean the rachitic animals

Copp They all had vitamin D The condition was one of low phos phoru rickets not low vitamin D rickets

Sobel You produced rickets in the presence of vitamin D?

Copp Yes

Sob l How much vitamin D did you give?

Copp There were 8 units per gram of diet and since the rats consumed about 6 grams of diet per day they received about 50 units daily

Butler That is a big dose

Sobel It is a small dose

Harrison No it is a large dose

Copp It is a large dose

Follis Eight thousand units per kilogram of diet

Copp Yes 8 000 units per kilogram that is right It contains 8 units per gram that is a better way of putting it But as far as Dr Howard s effect goes the level of phosphate was so low that even after the administration of enough phosphate to bring the level in the serum to normal there was little possibility of complex formation in the filtrate We also gave other animals calcium sodium Versenate and we found as Dr Neuman reported that there was an increase in the calcium excretion which approached the filtration rate in both rachitic and normal animals

Conference Discussion

Follis It is interesting I think in relation to Schneider and Steenbock s observations that if you put more vitamin D in this diet you get renal stones

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Day H C and McCollum E V Mineral Metabolism Growth and Symptomatology of Rats on a Diet Extremely Deficient in Phosphorus *J Biol Chem* 130 269 283 (1939)

Schneider H and Steenbock H A Low Phosphorus Diet and the Response of Rats to Vitamin D *J Biol Chem* 128 159 (1939) Calcium Citrate Uroliths on a Low Phosphorus Diet *J Urol* 43 339 (1940)

Copp I would not be surprised The urine contained practically no phosphate in these animals but there could be calcium oxalate or some thing else

Shorr To what extent is the Versenate saturated?

Copp Well it is the calcium sodium salt prepared so that it contains equivalent amounts of sodium and calcium

Shorr I was just wondering whether there was any possibility that when Versenate gets into the blood it does something there to the calcium which then affects the capacity of the tubules to reabsorb the calcium

Copp The quantity of Versenate salt used was large and even on the basis of partition of the radiocalcium between the calcium of the chelate salt and that of the blood most of the Ca^{45} would be in the Versenate fraction

Handler Are these figures all referable to the glomerular filtration rate of 23 cc per minute per square meter?

Copp That value is put down for information We did not calculate it we looked it up in the literature All of the figures can be compared to that value though

Handler So that in rickets the calcium clearance is just about 50 per cent of the normal glomerular filtration rate?

Copp Yes

Rubin Were these clearances calculated on the ratio of the *serum* Ca^{45} to the Ca^{45} in the urine?

Copp They were all calculated on the basis of the Ca^{45} in the serum to the Ca^{45} excretion in the urine This is a lazy man s method of measuring clearance

Shorr Was the calcium sodium Versenate radioactive?

Copp The animals received a tracer dose of Ca^{45} intravenously and then they received calcium sodium Versenate intraperitoneally

Harrison Dr Copp your calculation would actually include the total calcium in the serum would it not?

Copp That is right

Harrison If we assume that half of the calcium in the serum is not filtrable the clearances would indicate that all of the filtered calcium appears in the urine and there is no tubular reabsorption of calcium under these conditions

Copp No because the calculation is based on the whole serum

Harrison But the Ca[4] would be partitioned between the calcium bound to protein and the ultrafilterable calcium and the specific activities of both fractions of the serum calcium would be the same

Copp Yes that is right you are quite right

Harrison If that is true the calcium clearances are equal to the assumed filtration rate

Neuman Is not the filtration rate in the same terms?

Copp Yes it is in the same terms

Harrison Those are not your own determinations?

Copp No they are not However I might point out that I have seen values from 17 to 36 cc/min/sq m for the filtration rate reported by these same workers

Howard It really shows a perfectly colossal effect by vitamin D on the renal excretion of calcium

Copp Well no

Howard Everybody who has ever reported on the amount of calcium that comes out in the urine in vitamin D deficient rickets has commented on the fact that no calcium comes out at all

Follis Dr Copp is discussing a very particular kind of rickets

Copp Yes it is a very particular kind of rickets in a very special kind of animal

Follis It is a phosphorus deficiency rickets

Howard Well vitamin D deficiency rickets is phosphorus deficient rickets too isn t it?

Follis Aren t these experiments at variance with McCollum s metabolic studies?

Copp No He found a very high excretion of calcium in his phosphorus deficient rats even in the presence of vitamin D [3]

Follis Was the excretion from the urine or the gut?

Copp It was in the urine and he commented upon that

Harrison But even in the absence of vitamin D on the ordinary Steenbock diet there is a very high excretion of calcium in the urine

Copp Yes in the rat

Harrison In other word rickets in the rat is quite different from that in human beings in that it is always associated with a high excretion of calcium in the urine

Howard Which is the opposite of the human being

Harrison Exactly

Copp I think these states may not even be the same condition We call all these conditions rickets because the bones all look the same

Sobel The question is would a rachitic rat that has not received vitamin D behave the same way? Have you any information?

Copp No we have not—oh yes we did study rats prepared on the Steenbock diet They show the same high urinary excretion of radiocalcium and calcium But we do not have clearance figures for them

Butler Did you look at the parathyroid glands of these rats?

Copp No we did not However we have given parathyroid extract to these rats and it had no significant effect

Kramer What was the calcium phosphorus ratio there?

Copp About 30 to 1

Bartter Dr Copp do you have any data on the serum calcium level of these animals?

Copp It was between 10 and 11 mg per 100 cc The serum phosphorus concentration was from 1 to 3 mg per 100 cc The rat normally has a serum phosphate level of about 8 mg per 100 cc I think

Harrison This was probably a high alkaline ash diet was it not?

Copp Yes it was

Harrison Dr Shorr do you think that these animals would be excreting large amounts of citrate? That might influence the calcium excretion

Copp Yes that is right

Shorr We have noticed that it is not the citrate that seems to determine how much calcium is excreted but rather that the extent of the calcium excretion determines the magnitude of citrate excretion

Harrison But citrate can complex calcium in the urine and according to Dr Neuman any complexing agent may interfere with the tubular reabsorption of calcium and thereby increase the urinary excretion of calcium

Shorr My only reason for doubting this explanation is based on the experiments we have done in which we gave intravenous citrate daily for long periods with very large increases in the excretion of citrate and no change in the calcium content of the urine

Rubin Citrate is a poor complexing agent from this point of view it is metabolized and it is handled by many mechanisms to which some of these other agents are not subject

Shorr Yes but it does come out in the urine in excess under these conditions without influencing the calcium excretion

Harrison Dr Shorr in your studies was the urine pH acid?

Shorr I do not remember

Sobel Would the increase in citrate be sufficient to account for such a change

Shorr As I said the essence of our findings was that the citric acid excretion increased during its intravenous administration without influencing the excretion of calcium

Sobel It almost would appear that a certain amount of serum phosphate protects against calcium loss because maximal calcium loss seems to occur when the serum phosphate concentration is extremely low

Copp Well as I pointed out if you raise the serum phosphate level the high excretion of Ca^{45} disappears immediately

Partier The animal had hypercalcemia didn't they? Did the hypercalcemia disappear when you raised the serum phosphate level?

Copp The rachitic rats were not very hypercalcemic The serum calcium level was only 10 or 12 mg per 100 cc When you give phosphate intraperitoneally the serum calcium falls to 6 or 7 mg per 100 cc and the calcium and Ca^{45} disappear from the urine

Kramer I assume that the rickets heals when you add phosphate?

Copp Yes it does

Kramer And probably the calcium is then deposited with the phosphorus in the bone

Copp Yes

Sobel It is possible that the mechanism of absorption requires calcium phosphate for the formation of organic phosphate and when the phosphate is not present organic phosphate cannot be produced?

Butler Do all the experts of rat renal physiology agree that the glomerular excretion rate per square meter is only 23 cc per minute? That to me is just unbelievable in my ignorance I admit

Handler It would make the filtration rate much lower per unit of surface than it is in the adult man which is hard to believe One would expect quite the opposite if anything

Copp The factor is 10 per square meter

Butler Are you sure you corrected it to make it 23 cc per square meter and not per the whole rat?

Copp Yes The value given was 0 23 cc per minute per 100 square centimeters or 23 cc per minute per square meter However it should be pointed out the values as high as 36 to 40 cc per minute per square meter have been reported

THE EFFECT OF VITAMIN D ON CALCIUM ABSORPTION IN RATS WITH LOW PHOSPHORUS RICKETS

HAROLD E HARRISON

From the Baltimore City Hospitals Baltimore Maryland

Armstrong Dr Harrison would you like to present your material on the absorption of calcium?

Harrison In Dr Howard's introduction he considered the question of calcium absorption It may be of interest to discuss the effect of vitamin D on calcium absorption as determined by the feeding of Ca^{45} to rats with low phosphorus rickets

In our experiments which have been published[5] 10 mg of calcium [illegible] calcium chloride containing Ca^{45} was given by stomach tube at intervals of 2 4 and 24 hours following intubation groups of rats were sacrificed and the amount of Ca^{45} remaining in the gastrointestinal tract plus that excreted in the feces was determined The percent of the administered Ca^{45} absorbed could then be calculated In Figure 48 the data are given as the percent of Ca^{45} absorbed at the indicated time and are the averages of groups of animals *The open circles* represent untreated rachitic rats *the solid circles* rats given vitamin D prophylactically (100 units per week) and *the open triangles* rachitic rats treated with 3 000 units of vitamin D 72 hours before the administration of the Ca^{45}

Vitamin D and Absorption of Radiocalcium

The data indicate that in all groups of animals there is an initial rapid intestinal absorption of Ca^{45} Up to 4 hours after intragastric administration of the calcium solution there are no differences in the rate of absorption among the three groups of rats After 4 hours however a difference is apparent between the calcium absorption of the untreated rachitic rats and of the vitamin D treated animals The untreated rachitic rats show no further absorption of calcium after the 4 hour interval whereas in the vitamin D treated rats absorption of calcium continues so that by 24 hours after the feeding of the calcium chloride solution the percent of Ca^{45} absorbed is significantly greater in the rats given vitamin D Similar results

[5] Harrison H E and Harrison H C Studies with Radiocalcium The Intestinal Absorption of Calcium *J Biol Chem* 188 [illegible] (1951)

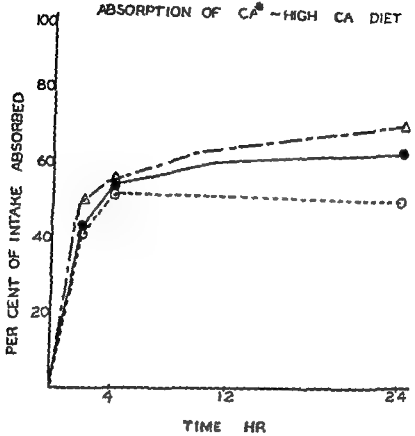

Fig 48 The Effect of Vitamin D on the Intestinal Absorption of Calcium by Fed Rats with Low Phosphorus Rickets as Measured with Radiocalcium

All of the rats were maintained on a high calcium low phosphorus rachitogenic diet At 0 time 10 mg of calcium containing Ca^{45} was given as a solution of $CaCl_2$ by stomach tube *The open circles* represent untreated rachitic animals *the solid circles* represent rats given vitamin D prophylactically and *the open triangles* represent rachitic rats given 3000 units of vitamin D 72 hours before the start of the experiment

with respect to the effect of vitamin D have been reported by Lindquist[168] although his data differ in certain details

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[168] Lindquist B Effect of Vitamin D on the Metabolism of Radiocalcium in Rachitic Rats *Acta Paediatrica Uppsala* 41 Supp 86 (1952)

Two Phases of Calcium Absorption

In these experiments the absorption of calcium can be separated into two phases. There is an initial rapid rate of absorption followed by a period of absorption at a slower rate. The initial rapid absorption may be due simply to diffusion of the calcium from the intestinal lumen into the body fluids dependent on the high concentration gradient existing at this time. During this phase Ca^{45} is being absorbed from the proximal portion of the small intestine.

Cutman: What portion of the gastrointestinal tract did you intubate?

Harrison: The solution was put into the stomach.

The second phase of calcium absorption represents absorption from the distal intestine. The pH of this portion of the intestine is high enough so that the concentration of calcium in solution is probably very low and absorption may at this stage depend on an active transport mechanism requiring cellular activity. It is this phase of calcium absorption which apparently is increased by vitamin D.

Armstrong: How many animals do you have to have in order to get a reliable statistical difference?

Harrison: There are ten animals in each group.

Kramer: Did you analyze the intestinal contents as a whole or did you divide it up?

Harrison: The intestinal tract was divided into portions the contents of which were analyzed separately. At the 4 hour interval the stomach and the proximal small intestine were almost free of Ca^{45}. The unabsorbed Ca^{45} was in the distal third of the small intestine and a considerable portion was already in the large intestine. A similar separation of calcium absorption into two phases has been found by Carlsson, with the slow phase also occurring when the Ca^{45} was chiefly in the distal small intestine and in the large intestine.

Kramer: At the end of four hours?

Harrison: Yes.

Carlsson, A. On the Mechanism of the Skeletal Turnover of Lime Salts. *Acta Physiol. Scand.* 26: 200 (1952).

The Effect of Feeding on Calcium Absorption

Shorr Were the animals fasted during this particular period?

Harrison The animals were not fasted i e they were allowed food through the night but on the morning of the experiment the food was removed If the rats were fasted or fed a low calcium diet before the experiment a different picture was seen (Figure 49) In this figure the absorption of Ca^{4} given as in the preceding experiment was determined in rats which had been *fed a calcium free diet for 72 hours or fasted for 24 hours so* that the intestinal tract was almost free of calcium before the administration of the Ca^{45} In this series no difference is seen between the rachitic rats and the vitamin D treated rats in the rate of absorp ion of Ca^{4} This is compatible with the idea that calcium is rapidly absorbed when given in a *soluble form and in this phase of absorption vitamin D is not necessary* In the first series of experiments the Ca^{45} mixes with the insoluble dietary calcium *and the absorption of* Ca^{4} *measures the rate of absorption of* the total calcium The experiments suggest that under conditions in which the calcium in the intestinal lumen is present in an insoluble form vitamin D is apparently necessary for maximum absorption of calcium whereas maximum absorption can occur in the absence of vitamin D if the calcium is present in a soluble form

Shorr Do you think it is a dilution factor that if you added some nonabsorbable bulk material you might dilute it?

Harrison Yes

Shorr That is the calcium in the first set was mixed with the residue of the food?

Harrison That is correct it was mixed with food residue containing large amounts of calcium carbonate so that in the first set of experiments we were studying absorption of administered calcium chloride plus residual dietary calcium carbonate

Shorr But actually the calcium content of the mixture that was exposed to the large intestine was greater in *Series A* than in *Series B* and yet there was less of the radioactive calcium absorbed is that correct? Could there have been just as much total calcium ab orbed and less of the radioactive because of dilution?

Kramer You are talking of percentages?

Harrison Yes the percentage of the administered Ca^{45} We are not talking of absolute amounts of calcium

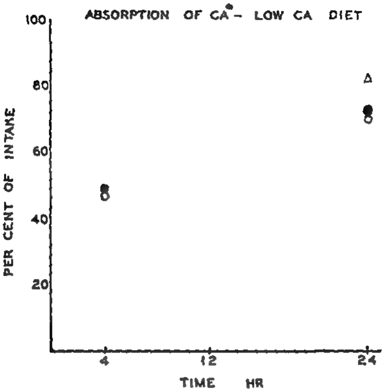

Fig 49 The Effect of Vitamin D on the Intestinal Absorption of Calcium by Fasted Rats with Low Phosphorus Rickets as Measured with Radiocalcium

All of the rats were maintained on a high calcium low phosphorus rachitogenic diet. The animals were placed on a calcium free diet for 3 days and then fasted for 24 hours before the start of the experiment in order to empty the intestinal tract of calcium. At 0 time 10 mg of calcium containing Ca^{45} was given as a solution of $CaCl_2$ by stomach tube. *The open circles* represent untreated rachitic animals; *the solid circles* represent rats given vitamin D prophylactically; and *the open triangles* represents rachitic rats given 3000 units of vitamin D 72 hours before the start of the experiment.

Shorr But you may be getting just as much total calcium absorption

Harrison That is right. We were trying to determine how vitamin D functions in the absorption of calcium.

In the latter set of experiments in which the intestine was free of calcium before the test dose of calcium chloride was given no effects of vita

min D on absorption were observed similar to those reported in other types of studies

Handler But are these differences of a greater order?

Harrison No not in the rat

Stearns When you have a depleted animal the higher the intake the higher will be the absorption

Harrison Yes

Exchange of Radiocalcium Across the Intestinal Wall

Handler Since your assay is entirely in terms of radioactive calcium to what extent would you be misled just by exchange? The amount of material in the gut might be quite different in absolute amounts when equilibrated

Harrison We studied that problem before starting the absorption experiments Calcium with a specific activity of approximately 1 000 counts per second per mg of calcium was administered to rats which had been on a calcium free diet for 72 hours so that their intestinal tracts were freed of calcium At the end of 4 hours the calcium remaining in the lumen of the small intestine was isolated and the specific activity was found to be about 900 counts per second per mg of calcium This decrease could be explained by dilution of the administered calcium with the amount of calcium which might have been secreted with intestinal juices into the intestinal lumen during the 4 hour period There is no evidence of any rapid exchange of calcium across the intestinal wall since at the time that the specific activity of the calcium in the intestinal lumen was about 900 counts per second per mg the specific activity of calcium in the serum was less than 150 counts per second per mg We concluded that the disappearance of radioactive calcium from the intestine represents absorption of calcium

Conference Discussion

Handler It is a one way passage

Bartter Is not it right to say that a dilution effect might produce the difference in slopes between Figures 48 and 49 but that it would not account for the individual differences (between treatments) in Figure 48?

Harrison Yes you might expect the absorption rates in these two groups of experiments to have somewhat different slopes but the significant point seems to be that in one set of experiments an effect of vitamin D was observed which was not found in the other type of experiment

Shorr Were phosphate determinations made on the washings in the respective animals?

Harrison Yes

Shorr What did they show?

Harrison There was considerable variation in phosphate absorption

Shorr I meant the total phosphate in the fasted animals

Harrison In the fasted animals the phosphate in the intestinal contents was low

Shorr And in the animals fed was there evidence of a contribution by the inner circuit of phosphate?

Harrison Well there was a considerable amount of phosphate in the intestinal contents I am not sure that I understand your question

Shorr Was there any difference between the amount of phosphate in the final contents in the rachitic animals receiving D and those not receiving D? I was just considering the phosphate as a possible factor that tied up calcium

Harrison I do not believe that the actual amount of phosphate in the intestinal contents differed greatly between the rachitic and the vitamin treated rats

Shorr And the pH changes?

Harrison We did not do pH determinations This subject has been reinvestigated by Steenbock and his associates [1] They found in confirmation of older studies by Zucker and Matzner [2] that vitamin D given to rats on a rachitogenic diet does influence the pH of the intestinal contents In the distal intestine of the vitamin D treated rats the pH is somewhat lower than in the untreated rachitic animals It is in this portion of the intestine that the absorption of calcium seems to be influenced by vitamin D in our studies

Park Would you say that the vitamin D effect might have occurred when radioactive phosphorus was given in conjunction with food?

Harrison Yes I think so

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[1] Steenbock H Bellin S A and Wiest W G Vitamin D and Urinary pH *J Biol Chem* 193 843 (1951)

[2] Zucker T F and Matzner M J On the Pharmacological Action of the Antirachitic Active Principle of Cod Liver Oil *Proc Soc Exp Biol and Med* 21 186 (1924)

Shorr You might eliminate the phosphate factor by the use of aluminum gels

Handler What is bothering me is that while there is no doubt that the data in Figure 48 show *statistically significant* differences are these *biologically meaningful* differences in the sense that they can be a real etiological factor in the development of rickets The presence of a lot of food with the calcium would completely eliminate your two hour effect so that your entire scale would be dropped and then you would have a 50 per cent difference instead of what looks like a 5 per cent Is that what you were thinking about?

Harrison It is one point

Handler Of course rats are difficult animals

Harrison Yes rats are not the best animals for study of vitamin D effect since they *do not develop manifestations of vitamin D deficiency unless* the phosphorus intake is low and the calcium to *phosphorus* ratio of the diet is high We cannot translate the magnitude of the differences observed into studies on man The differences found between the vitamin D treated and the rachitic rats with respect to the absorption of Ca^{45} are of the same order of magnitude as the differences found by Nicolaysen[17] in studies of the effect of vitamin D on the absorption of calcium from intestinal loops of rats

Gutman As I understand it the point you make is this in the stomach and immediately adjoining the duodenum where the medium is acid absorption of calcium is rapid and marked and the vitamin D intake makes *no significant difference In the more distal portions of the intestinal tract* where the pH becomes more alkaline vitamin D does exert a significant effect by accelerating and increasing the absorption of calcium which without vitamin D might be slow and incomplete

Harrison Yes that is right

Gutman Do you imply that the accelerating effect of vitamin D on calcium *absorption in the alkaline portions of the gut* operates through some active (enzymatic) transfer mechanism?

Harrison *I am suggesting that there are two processes involved in the* absorption of calcium one of which is possibly a simple diffusion of calcium due to a concentration gradient The second process may be an active absorptive mechanism requiring cellular activity Vitamin D may be neces

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[17] Nicolaysen R Studies upon the Mode of Action of Vitamin D III The Influence of Vitamin D on the Absorption of Calcium and Phosphorus in the Rat *Biochem J* 31 122 (1937)

sary in this second transport mechanism It is also true that a pH change which permits calcium to remain in solution would facilitate absorption by diffusion

Passett Are these young animals?

Harrison Yes all very young animals

Passett That is why you have the very high rate of absorption

Harrison Yes there is a marked age difference These rats were 6 weeks of age at the time of the experiment At 3 months of age the rate of absorption can be shown to be lower than in the younger rats This again confirms the studies of Nicolaysen who found similar age effects

Kulm May I show an illustration bearing on this point The chart (Figure 50) covers the earlier section of Dr Harrison s data The animals are rabbits which have fasted for 24 hours Calcium is injected in tracer quantities directly into the upper intestine and the upper part of the figure shows calcium injected a calcium chloride into the intestine and measured in the blood The significant point is that the activity in the blood is exactly the same as you would obtain if you injected the same dose of radioactive calcium in the blood in other words you must come to the conclusion that the absorption of the tracer quantities under these conditions is total and complete in these animals

On the other hand we have done the same experiment using an injection of calcium citrate and an injection of calcium Versenate and the blood level are lower This does not mean however that the absorption is any less It indicates rather that it takes a while for the equilibrium by exchange to occur inside the intestinal tract so that the radioactive calcium is then available for absorption We know that to be the case with Versenate because we know that the material is not absorbed Using carbon tagged Versenate we know that it goes right through the intestinal tract unchanged and unabsorbed Therefore the calcium originally bound here must have found its way into the blood after it had been released by free exchange in the intestinal tract This would argue that the exchange process took a little while It does not argue that the absorption is any lower

Harrison In this case the exchange can be due to the entrance of stable Ca^{4} into the intestinal tract which enters into combination with the Versenate by exchange with the Ca^{45} Exchange in this direction can occur because extracellular calcium enters the intestinal lumen in the intestinal juices

[17] Nicolaysen R The Absorption of Calcium as a Function of the Body Saturation with Calcium *Acta Phys Scand* 5 200 (1943)

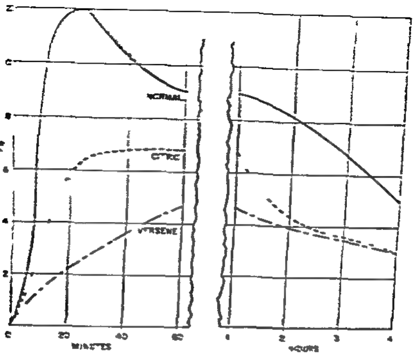

Fig. 50. The Effect of Complexing Agents on the Gastrointestinal Uptake of Tracer Quantities of Radioactive Calcium in Rabbits.

Sachs: The ... right, and the more rapid ... ability of calcium bound by citrate is a reasonable explanation ... or to ex... one of the higher ... concentr... in equili... with citr... and ... more radio... available ... for the ... But the ... were ... we saw here was th... immedi... tracer given into th... a ma... er o...

Armstrong: ... the inte... you radioactive cal...

Rubin: I am ... but beyond the pylorus

Shorr: With a c...

Rubin: The solut... for no good rea on this pH

Neuman The solution has practically no buffering action at that pH Citrate will not buffer effectively above pH 6 5

Rubin Yes that is correct

THE PROBLEM OF ADAPTATION TO LOW CALCIUM INTAKES

ROBERT A McCANCE

From the Department of Experimental Medicine
University of Cambridge Cambridge England

Armstrong Dr McCance has chosen to speak about The Problem of Adaptation to Low Calcium Intakes Please proceed Dr McCance

The Case of a Vegetarian with Osteomalacia

McCance I was invited to see a patient some months ago by Dr Charles E Dent She was a girl of twenty four years a strict vegetarian and in the habit of eating 1 lb of Hovis (brown) bread per day She had been a vegetarian all her life but for the last six years her diet had contained no milk cheese or other animal products and probably very little vitamin D About eighteen months before she had had a fall on her right hip This was followed by some stiffness and pain on attempting to get up from a chair and to start walking She was seen by one or two doctors and was admitted to several hospitals but she was a difficult patient and discharged herself on each occasion before a firm diagnosis was made She treated herself for a time with about a gram of calcium per day in tablet form and 500 units of vitamin D This made her feel better but apparently she did not persist with this treatment

When she was seen by Dr Dent she presented the symptoms and signs of osteomalacia Her pain and her bones were typical The latter were extremely decalcified and contained multiple pseudo fractures of the Looser Milkman type The serum calcium level was 8 3 mg /100 cc the inorganic phosphorus concentration 1 2 mg /100 cc and the alkaline phosphatase level 39 3 King Armstrong units The rest of the findings are immaterial Dr Dent considered her in all probability to be a person who had become highly resistant to vitamin D and he gave her 10 000 units of vitamin D per day as a pilot dose and 2 grams of additional calcium After four days of this treatment her serum chemistry began to return towards normal and in ten days it was normal

Her clinical condition did not improve as rapidly but in less than three weeks there was a suggestion that the pseudo fractures were beginning to fill up Calcium balances were carried out before the treatment was started and six days after the treatment had d (Table XXII) They

TABLE XXII

The Calcium and Phosphorus Balances Before and During Treatment with Calcium and Vitamin D in a Patient with Osteomalacia

	Intake	Excretion			Balance
		Urine	Feces	Total	
	(gm/24 hr)	(gm/24 hr)	(gm/24 hr)	(gm/24 hr)	(gm/24 hr)
CALCIUM					
Before treatment	0.66	0.01	0.69	0.70	−0.04
During treatment	2.88	0.09	1.72	1.81	+1.07
PHOSPHORUS					
Before treatment	0.87	0.49	0.39	0.88	−0.01
During treatment	0.87	0.33	0.31	0.64	+0.23

showed that before the treatment she was in slight negative calcium balance but after the administration of the vitamin D and calcium she began to have positive balances of the order of 1 gram of calcium per day. In six weeks she was symptom free.

Poor Absorbers of Dietary Calcium

Why did she become so decalcified? Looking at it in one way there is no problem. Our experience in 1942 was that negative calcium balances were the fate of most of the experimental party (myself included) when we had diets like that of this girl which contained a lot of brown bread and not much calcium.[34] The individuals in our party varied greatly in their ability to absorb calcium from a given diet and I concluded in the first place that this lady was by nature one of the poorer absorbers and had become decalcified for this reason but against this I had to set her excellent response to treatment with calcium and vitamin D.

Incidentally I always have been interested in the symptomless way in which some people, this girl for instance, become decalcified. I am a very poor absorber of calcium myself and if I go on to a high phytic acid low calcium diet (Tables XXIII, XXIV) I get tetany.[35] This is so un

[34] McCance, R. A., and Widdowson, E. M., Mineral Metabolism of Healthy Adults on White and Brown Bread Dietaries, *J Physiol* 101 44 (194)

[35] McCance, R. A., and Widdowson, E. M., The Energy Value of Oatmeal and the Digestibility and Absorption of Its Proteins, Fat and Calcium, *Brit J Nutrition* 221 (1948)

McCance, R. A., and Walsham, C. M., The Digestibility and Absorption of the Calories, Proteins, Purines, Fat and Calcium in Wholemeal Wheaten Bread, *Brit J Nutrition* 2 (1948)

TABLE XXIII

The Calcium Balances of Subject R A M on Different Diets

	Intake	Excretion			Balance
		Urine	Feces	Total	
	(gm./24 hr.)	(gm./24 hr.)	(gm./24 hr.)	(gm./24 hr.)	(gm./24 hr.)
White bread diet	0 72	0 22	0 54	0 76	—0 04
Diet containing bread made from 92% extraction flour	0 68	0 17	0 66	0 83	—0 15
Diet consisting of bread made from 100% extraction flour	0 49	0 22	0 54	0 76	—0 27
Oatmeal diet	0 73	—	0 95	—	—

pleasant that I would never go on with the diet long enough to get osteo malacia The bone calcium of some people must be more labile than mine and more readily set free by parathyroid hormone to keep up the serum calcium level This is really a side issue to my main problem but it seems important to remember that there are these individual differences in peo ple s ability to absorb calcium from a given diet and in their response to a negative calcium balance

Survival of Populations on Low Calcium Intakes

Against the behavior of this girl of Dr Dent s we have to place the fact that the greater part of the populations of India Africa and other parts of the world manage to survive on diets containing much phytic acid and very little calcium diets on which most people in Britain and the United States would almost certainly go into a negative balance

Butler What was her calcium intake?

McCance It was about 0 66 grams a day when she was in the hospital We do not know what her calcium intake was when she was living on her own self chosen diet outside except that it was a good deal lower than that

Bartter Did she expose herself to sunlight for any reason or not at all?

McCance Not particularly but she was up and about and leading an active life

TABLE XXIV

Calcium Balances on Different Diets

	Level	Intake	Excretion			Balance	Bread Eaten
			Urine	Feces	Total		
		(gm./24 hr.)	(gm./24 hr.)	(gm./24 hr.)	(gm./24 hr.)	(gm./24 hr.)	(gm./24 hr.)
McCance Experiments							
White bread	Avg	0.50	0.18	0.34	0.52	−0.02	460
	Max.*	0.72	0.25	0.54	0.76	+0.05	700
	Min.*	0.38	0.12	0.19	0.39	−0.12	—
Brown bread	Avg	0.56	0.15	0.50	0.65	−0.09	460
	Max.*	0.68	0.22	0.66	0.83	−0.01	700
	Min.*	0.50	0.10	0.36	0.54	−0.19	—
McCance and Walsham							
Wholemeal Canadian and English together	Avg	0.40	0.16	0.44	0.60	−0.20	1040
	Max.*	0.55	0.22	0.66	0.81	−0.12	—
	Min.*	0.32	0.11	0.27	0.43	−0.27	—
Oatmeal	Avg	0.51	—	0.82	—	—	—
	Max.*	0.72	—	1.01	—	—	—
	Min.*	0.20	—	0.45	—	—	—
German Civilians Undernourished							
High extraction German bread	Avg	0.83	0.20	0.77	0.97	−0.14	750
	Max.*	0.92	0.29	0.96	1.18	+0.05	—
	Min.*	0.66	0.11	0.60	0.77	−0.30	—
German Children							
High extraction German bread	Avg	0.75	0.08	0.59	0.67	+0.08	600
	Max.*	0.80	0.12	0.68	0.78	+0.25	—
	Min.*	0.67	0.03	0.41	0.48	+0.05	—
High extraction German bread and added Calcium	Avg	2.48	0.13	2.01	2.14	+0.34	600
	Max.*	2.89	0.19	2.47	2.60	+0.51	—
	Min.*	1.99	0.07	1.48	1.58	+0.22	—

*Note that the maximum (and the minimum) figures for intake, urine, feces, total excretion and balance are independent values with no correlation to each other because they are the extremes of variation in each series of experiments.

The question is how do these populations survive? How do the children ever grow on these diets and how do the adults maintain calcium equilibrium? As Nicholls and Nimalasuriya[175] pointed out many years ago and as Hegsted Moscoso and Collazos[176] have re emphasized recently the children do grow they do not have rarefied bones and although they seldom grow as tall as well nourished Europeans and Americans there is no evidence that their unfavorable calcium and phosphorus intakes are the cause. There seems no doubt that these native populations are able to absorb enough calcium from these diets to enable them to grow reasonably well and to calcify their bones. How do they do it? Would Americans adapt to diets such as these and if so how soon and to what extent?

Adaptation to Low Calcium Intakes

Walker Fox and Irving[177] have claimed that their subjects calcium balances improved after a short time on such diets and that thereafter they no longer lost more calcium than they ingested. Against this must be set the fact that six out of seven undernourished men studied by us in Germany[178] were in negative calcium balance on the diets they were eating at that time although they had been having these large amounts of brown bread and very little calcium for at least two years before being seen by us *(Table XXIV)* *Perhaps two years was not long enough for them to* adapt but the evidence of Walker and his associates is not very convincing. Walker had but three subjects himself and two others and they all appear to have been particularly good absorbers of calcium before the experiment began. Nicolaysen and Njaa's[179] experiments on adaptation in adults also are unconvincing. To put it bluntly we ourselves have obtained no evidence of adaptation in adults and are dissatisfied with the evidence to this effect which has been produced by others.

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[175]Nicholl L and Nimalasuriya A Adaptation to a Low Calcium Intake in Reference to the Calcium Requirements of a Tropical Population *J Nutrition* 18 563 (1939)

[176]Hegsted D M Moscoso I and Collazos C A Study of the Minimum Calcium Requirements of Adult Men *J Nutrition* 46 181 (1952)

[177]Walker A R P Fox F W and Irving J T Studies in Human Mineral Metabolism I The Effect of Bread Rich in Phytate Phosphorus on the Metabolism of Certain Mineral Salts with Special Reference to Calcium *Biochem J* 42 452 (1948)

[178]Widdowson E M and Thrussell L A The Ab ... l Excretion of Nitrogen Calcium Magnesium and Phosphorus Studi ... trition Wuppertal 1946 *) Spec Rep Ser Med* ... *Lond* No

[179]Nicolaysen R and ... stigations ... Phytic Acid on the Absorption of Cal ... s and ... *Scand* 22 46 (1951)

A n al exper ment m fa or of adaptat on are equall uncon nc ng Mellanl s dogs d l not adapt s ffc entl to pre ent the r bones fron be com ng decalc fied by lo calc m l ets r ch n pl yt c ac d and conta ng ery l ttle v tan n D It ha been suggested tl at adap at on can take pla e n a ch ld n a few lavs [8] but h ldren may be n a d fferent categor and a orb calc um n l better tl an adult A group of s x German ch l dren ve tud ed howe er abs rbed only 160 m[g] of c l u a d[a] and reta ne l 80 n g on cal um ntakes of 750 g l cal n to phospl or s rat os of 0 3 (Table XXIV) Tl s doe not eem er m l an l the cal un ntake as ery l l gl er tl an e a e led to l el e e tl e case among man nat e pop lat on A otl er lar group of cl l lren l n[g] n the same n pl anage and ea n a n lar d et l ut l o l a l had cal un ca l om e ad led o the r bre l for the pa t s x mo t so tl at tl e r cal c um ntakes were 500 n g n la al orbed 4 0 n g and re a ne l 340 n g

Quant tative Aspects of Skeletal Rarefaction in Osteomalac a

I k w tl at tl e negat e bala es wh cl we l e f n l n o el e a l tl e unde our shed Ge nan ha e bee q ant at el q te n all and e ha n calc late l tl a tl ey ould ren e onl al o t 4 per cent of tl e l od s l n a ye r Dr Dent s pa en had ha l x ear n l cl to be e l cal fie l a d th was e d ntl enou l to p od ce ad anced te n ala a

I l r Sl e l ad no l urther?

M Ca c No n e at all

Baba a tz l as sa d tl at 30 t 50 per cent f t e bc ne cal n ha to l e ren o ed l ef re tl e ra efact on ca be letecte l ra l olog all Bal a antz s t a e ma be too l l for n he ca e h ch l l a n le c be l ra lo- lo[g] l n p r en nt a le e l le a ouple of eeks Of cour e n all ra l ol al cha es w ll be de ecte l n l re real ly f t o x m f tl e s n e p r at l fferet st es ere a a lat le f r on par n If o l la e l d og rapl l e bee t ken lecal f cat o t tl e n ucl m re e re l f re t ca be a l l erta t to l a e take pla

In p of tl e l e of tl e e lecal f cat n l er e l l o teo

M l l l A f N al K a Th Ff of S L a y Fa n B n s o d he N re m W iam and W k s Co Ba more (1950

H ff J ecn F And O an l N en C The Eff ct of I h t c N on Ab p of C k m a l Pho p ru III In Cl d n Bu h J 10 5 5 194

B n z l O ec pcro Kad I Ba f 16 7 1 (194)

malacia has been accepted as an accompaniment of the undernutrition that followed the first World War from diets which did not differ so very much from our experimental ones although the absolute intakes of calcium and vitamin D may have been smaller Nevertheless we would expect to find osteomalacia under these conditions only if people failed to adapt The native populations who have lived on unfavorable diets from the time they are weaned seem to have managed quite well and some of the other strict vegetarians also

Through Dr Meiklejohn I have found out more about the rigid vegetarian sect which we have in the United Kingdom He knows of one family of a father a mother and two children who are apparently well after years on this diet Some have succumbed to anemias and signs of subacute combined degeneration of the cord but only this girl so far as we know to osteomalacia

Summary : The Problems of Adaptation to Low Calcium Intakes

We seem to be faced with the problem of trying to reconcile a lot of evidence that is unreconcilable Is some of the evidence wrong? Are all the Singhalese and Indians and Africans supermen as far as calcium is concerned? Perhaps the poor absorbers went to the wall years ago and have been bred out It may even be unnecessary now to struggle to improve their calcium intakes! Should we think more in terms of vitamin D than of calcium and phosphorus?

I do not think we know the answers to these questions I certainly do not but that need surprise nobody Perhaps some of you do and if so I am certain you will tell us

Conference Discussion

Kramer What month of the year did the patient present herself for the first time?

McCance Let me see It was in the Spring But this decalcification must have been going on of course for a long time

Kramer Oh yes of course I thought perhaps it might have become acute

McCance I think it did become acute in the Spring I think it was about a year ago when she first put in an appearance

Bartter I think there may be a point about the Central and South Americans of some interest The question came up in the Pan American Sanitary

Bureau as to why there was not more calcium deficiency in these people whose diets were completely free of milk and cheese, until it was discovered that when they make their tortillas they leach the corn in calcium chloride to start with, and then beat it up. There is apparently a very high calcium content in the tortillas, and all of these people eat them.

Stearns: Don't they use limestone mortars to grind their corn, too?

Bartter: Yes, I think they do.

Stearns: So they obtain quite a bit of calcium there. But they lose the thiamin.

Park: Would part of the answer be because they eat leaves, too?

McCance: My information is that the calcium intakes are low, and the source of my information is Hegsted's paper.[178] He has done calcium balances on prisoners in Peru, some of whom had been in jail for many years; they were living on diets which contained a very small amount of calcium, and he found them all in calcium balance.

Butler: Did he analyze the diet or did he estimate the diet?

McCance: He analyzed the diet. I have a reprint of his paper here. It is in the *Journal of Nutrition* for February, 1952. He found all of his subjects in calcium balance, and he estimated that a daily calcium intake of the order of 160 mg. would enable them to remain so.

Howard: Was the calcium content of the water included in that intake?

McCance: The water was hard, and he included the amount of calcium derived from it.

Kramer: Were these calcium balances obtained with or without vitamin D?

McCance: There were no vitamin D supplements.

Bartter: The prisoners must have had a lot of sunlight, cracking stones, though.

McCance: Only some of them were allowed out of prison into the yard. Some were exercised and some were not. I saw him about this when I was in Boston, and he said that one of them was not a Peruvian at all. He was a German, and he was in just as good calcium equilibrium as were the others. Of course, if you go to any native population and study their calcium metabolism, you are bound to find the adults in balance. The only interest is the level at which they achieve balance.

Stearns: Some years ago I discussed the question of the marked differ-

ence in stature between the people of North and South India with a physician from Calcutta He told me that the reported differences in protein and calcium intake between the two groups were true The South Indians are not only short of stature but extremely finely boned The ratio of width to length in the long bones was much less than in Europeans—the average wrist width in Calcutta is 2 fingers In contrast the Sikhs who use milk are as heavy boned as Europeans It seems that generations of low mineral intake do lead to skeletal modifications

In our own work I have been struck with the fact that for instance a group of eight children of the same age and about the same height and weight may have the same intake but one child may be excreting 25 mg of calcium daily in the urine and another 125 mg and that relative difference will exist no matter what the intake is that is with a rise or a fall there is still a five fold difference in the amount lost through the urine in these two children It has seemed to me in these people who have existed on a very low calcium diet for many many generations it is quite possible that those whose endocrine balance was such that the urinary calcium was too high to maintain balance probably did not grow up to reproduce and therefore eventually they would die out The South Americans are heavier boned but they are short They are about three or four years below North American children of the same age in height even though they have a fair calcium intake That reduction of stature is partly due or it may be very largely due to the fact that they have a very low protein intake I think it is most unfortunate that in the countries where the calcium intake is low the protein intake is always low also These two factors seem to have a good deal to do with determining final stature of people I have heard that in Guatemala the 12 year olds are about four years retarded in height and the adult heights are much less than ours although their bony structure seems to be of the same general type They do not have the very fine structure of the Asiatics I do not know whether any of these observations help to answer the question

McCance I think they are of great assistance You certainly have helped by suggesting that it is possible that some of the poor absorbers have been bred out so to speak It is interesting also that the Singhalese should have such small bones I asked Hegsted particularly if that was the case in Peru and he said No Of course there is no doubt that there are people who are very poor absorber of calcium I am one You see I can never get into calcium balance on a calcium intake which may be quite adequate for other people I certainly cannot hold my own on a brown bread diet (Table XXIII) On whole wheat or oatmeal diets I had more calcium in the feces than there was in the food so that anything in the urine was a dead loss to me whereas other people in our experimental

partly at any rate were able to absorb some calcium from their food There is an enormous difference between individuals

Armstrong Dr McCance you referred earlier to phytic acid and indicated that the young woman patient had probably had a diet with a very high phytic acid content before she sought medical advice

McCance Yes she had

Armstrong Is it not a fact that some individuals at any rate can adapt to increased quantities of phytic acid in their food?

McCance Well that is the whole question Can people adapt? If for instance everyone in this room now were to go on a diet containing a lot of brown bread and very little calcium—

Armstrong I thought you had investigated this point

McCance Not on the members of this Conference! Our evidence is that you would all be in a negative calcium balance The question is how long would you continue to be in a negative calcium balance? That girl of Dr Dent's continued in a negative calcium balance until she got osteomalacia

Fartter When you put her in the hospital did you continue the high phytic acid intake at first in the control period?

McCance No she was on a hospital diet

Fartter Then there is no explanation for the fact that she had that huge calcium deficit when she needed calcium

McCance Within the limits of experimental error she was in calcium equilibrium on the hospital diet containing 0 66 grams of calcium a day

Stearns We found in studying children in early adolescence that those who had been poorly nourished for a period of years before the study had very poor or very inefficient gastrointestinal tract and that it took five to six months of good diet before they had very good retention whereas children who were in so called abundant health and whose diet had always been good showed far better retention of calcium and phosphorus

Handler There comes to my mind a correlation which may be completely meaningless The group here has been considering this woman as being in some sense comparable to certain folk in economically underdeveloped portions of the world who live on diets low in protein and calcium In those people however who develop Kwashiorkor and who are frequently corn-eaters it is quite common to find hemosiderosis and hemochromatosis apparently due to an excessive absorption of iron Now we

pretend we know something about the mucosal mechanism for absorbing iron but the mucosal block which ordinarily prevents the absorption of excess iron does not operate in corn eaters at least in Africa I wonder if the same phenomenon might not be relevant to calcium Why do corn eaters absorb unusually large quantities of iron? If they can do that perhaps they can also absorb large quantities of calcium If they do the one I have no objection *a priori* to their doing the other either

Bartter But in the condition we have been discussing the situation is just the opposite

Handler The people I was referring to absorb a large amount of iron and develop hemosiderosis

Bartter But the individuals described by Dr McCance *do not* absorb calcium

Handler The problem of these other folk who subsist on very low calcium intakes is that the children grow and the adults apparently must be in calcium balance At least they are not all osteomalacic so as Dr McCance said you know they must be in balance even without doing a balance experiment on extremely low calcium intakes which points to a remarkably efficient absorptive process The correlation I am suggesting is that a large number of people who have incredibly efficient techniques for absorbing iron may also be unusually efficient in absorbing calcium I do not know the answer I just introduce this thought for consideration

Follis The hemosiderosis may be due to a nutritional deficiency since large amounts of iron are found in the tissues of pyridoxine deficient animals swine for instance[183]

Rubin It could also be due to a deposition of iron because of some lack of protein synthesis and therefore because of some defect in the handling of iron once it is absorbed One thinks in this case of Wilson s disease and copper absorption in which recently it has been shown that the mechanism of copper transport is at fault due to a lack of copper binding protein in the serum

Handler These observations have been made on individuals who were only moderately anemic and in whom deficient protein synthesis was not manifest

McCance Kwashiorkor as I know it is a disease of infants

Handler True but it is now a rather generic term used rather loosely

[183]Follis R H Jr *The Pathology of Nutritional Disease* Charles C Thomas Publisher Springfield (1948)

to apply to a form of malignant malnutrition in the tropics in persons who live largely on cereals such as plantains, manioc, or corn. At one time it was thought to resemble pellagra. The name of the disease comes from the fact that such individuals frequently have depigmented hair.

McCance: Yes, I know. I really can claim to know a little about kwashiorkor because we have a member of our department working on the subject now in Uganda. It is true that these children, after weaning, do get large livers, but I was not aware that they had hemosiderosis. The adults in these countries certainly have abnormal livers. They get a form of sclerosis and they sometimes have primary hepatomas.

Follis: As a matter of fact, they have rickets in Uganda, don't they?

McCance: No, I do not think so.

Follis: I was told by one of the gentlemen who is studying there that they have it. They have rickets because of the density of the foliage. [Laughter]

Harrison: Dr. McCance, isn't there some reason to believe that in infants and children there is a difference in the amount of vitamin D necessary, the minimum amount, aside from the problem of resistant rickets? If that were to hold true in adults, it is quite conceivable that in your particular patient you were dealing with a woman who not only had a low calcium intake but who happened to have a somewhat larger requirement for vitamin D than that of the average adult (which is an unknown factor entirely).

McCance: That is the natural assumption. I think it is one explanation.

Harrison: In other words, if on her low calcium intake you had given her vitamin D in ordinary doses, not 10,000 but perhaps 1000 or 2000, it is conceivable that she might have had a positive balance.

McCance: She might have pulled around.

Harrison: I was struck by Liu's studies of osteomalacia in Chinese women[1] that they could produce positive balances of calcium even on a low calcium diet by administration of vitamin D. The calcium intakes were less than 300 mg. per day. Is that right, Dr. Park?

Park: Yes. Liu and his associates[1] reported that the usual level of calcium intake in the Chinese dietary is approximately 0.337 gram.

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[1] Liu, S. H., Chu, H. I., Hsu, H. C., Chao, H. C., and Cheu, S. H.: Calcium and Phosphorus Metabolism in Osteomalacia. XI. The Pathogenetic Role of Pregnancy and Relative Importance of Calcium and Vitamin D Supply. *J. Clin. Invest.* [illegible] (1941).

Harrison This patient described by Dr McCance may have suffered from a relative lack of vitamin D perhaps due to an increased need for this vitamin beyond the normal requirements

McCance That is right and that is the point I tried to make We have to think here in terms of vitamin D and to think also in terms of calcium intake Would some of the people who were talking about the available and mobilizable calcium in bone be able to tell us how much calcium would be available to maintain serum calcium over a period of years?

Neuman I do not know and I have no information but as a guess I should think that over a period of years nearly all of the bone might be involved I refuse to accept at the moment anyway a solubility product and I believe therefore bone will dissolve whenever there is a fall from previously established equilibrium values If the calcium value fall some of the solid that has previously been deposited will dissolve If the level continues to fall more and more solid will dissolve As the available bone disappears then I should expect osteoclastic activity would open up new areas making them available to the circulation You can get severe osteomalacia You do get it

Reifenstein However Dr Neuman if bone is dissolved and osteoclastic activity opens up new areas in order to supply calcium to maintain the previously established equilibrium levels of calcium in the body fluids the usual histologic appearance of the bones is that of osteitis fibrosa not that of osteomalacia

Neuman Dr McCance there is one question about your patient Am I right that the calcium level was essentially normal?

McCance In the serum?

Neuman And the phosphorus was very very low and yet this was a low calcium diet?

McCance This girl had low calcium and low phosphorus levels in the serum

Kramer I would like to ask Dr Neuman why is it if the calcium is so readily mobilizable that a child can have a low serum calcium level for a long period of time go into tetany and die of convulsions when this tremendous pool of calcium could save the child by restoring the plasma calcium level?

Neuman The level has been low for a long time?

Kramer Well not necessarily It could be a long time it could be a short time

Sobel It could be a week only

McCance You should have seen the Chvostek sign I had after being on a brown bread diet for a fortnight and my bones were not doing anything to stop it

Henneman Did you have hypocalcemia too

McCance It happened on a Sunday unfortunately I had been on this diet for a fortnight and on the Saturday going home I began to have very bad cramps in my fingers particularly The interesting point is that they were quite indistinguishable clinically from the cramps you get with salt deficiency I could not tell them apart and I have had salt deficiency cramps I was stupid enough not to realize the obvious cause of these cramps I spent a very uncomfortable evening because I had to write two letters and of course the moment you start to write with this kind of cramp your fingers cramp on the pen You cannot let go of your knife and fork either I went to bed and I slept more or less all night When I got up the next morning I started to shave As soon as I touched my face with the razor I discovered that I had an absolutely magnificent Chvostek sign As it was Sunday morning I treated myself clinically rather than scientifically and took all the milk I could find the next day my serum calcium was of the order of 9 and I was much better I was not having cramps the next day

Neuman I want to hasten to interject here that while we know of a number of the processes governing the blood bone equilibrium we do not know how many still exist that we know nothing about I am thinking particularly of the organic inorganic interrelations The electron micrograph pictures that we saw put the crystal directly on the fiber In addition we know that the cement substance is in very intimate contact with the crystals Certainly these factors may limit the physicochemical processes that take place

I think we are getting back to the original point of difference between Dr McLean and me that is the true story lies somewhere in between an extreme emphasis on the physicochemical and an extreme emphasis on the cell It is a very complicated system in which the cell can alter the organic medium and thereby affect the physicochemical events and the physicochemical processes can take place to a limited degree without any intervention of the cell but only to a limited degree In our hydration studies even in those areas of bone where we know exchange takes place and therefore there is still some physicochemical interaction the degree of hydration was much smaller than one would predict on the basis of the crystal size We had in effect a certain number of ions that were not behaving in their free and easy physicochemical way The organic phase probably has some

thing to do with this This is speculation only and it is a mighty poor substitute for facts

Armstrong Dr McCance do you remember whether Dr Dent's patient had any disturbance of acid base balance which when corrected might have contributed to her skeletal recalcification

McCance No in that way I think she was perfectly normal She had normal stools There was no steatorrhea

Armstrong Would you mind indicating what you know about the state of your own skeleton? You have mentioned yourself as a peculiar subject who apparently easily develops a state of low calcium tetany Do the roentgenograms of your skeleton show anything unusual?

McCance No I think they are fairly normal

Albright How about doing a bone biopsy?

McCance You can take one if you like if you can arrange to have it done before *The Queen Mary* sails the day after tomorrow

Follis What are the variations you have run into with respect to calcium in order to keep the members of your group in balance? There were variations which were quite extreme weren't there?

McCance The variations were quite large and the interesting point is how constantly they maintained exactly the same relationship one to the other In our party of six or eight there was one man a Spaniard who was always by far the best absorber of calcium and I was always the worst It was the same week after week There was a real biological difference between the two of us I am certain that there is a real biological difference between every one of you here Just as people's faces are different so their calcium absorptions are different and probably everything else If you had worked extensively with metabolic experiments as I have you would know that even people's feces for instance are characteristically different When we were doing these metabolism experiments one look and I could have told you whose feces they were [Laughter] This is an absolute fact

Howard But it is more a matter of bones isn't it? Your bones do not support the blood levels the next fellow's do One fellow gets diarrhea and is in tetany within a week the next one does not get tetany for months in spite of the diarrhea And yet the latter individual has lost enormous amounts and the first fellow has not It still comes back to the bone I am not convinced that vitamin D does not have something to do with it because I was told that Dr Rhoads who studied sprue in Puerto Rico never saw anyone with tetany whereas in Baltimore 50 per cent of the people who

animals to maintain the concentrations of calcium and phosphorus at normal levels during fasting[185]

Albright Dr Park were the bones different in the two groups of animals you have just described?

Park Well the experimental animals had rickets The controls receiving ultraviolet light did not

Albright I was wondering whether in those cases of rickets where the blood values are less stable one finds the trabeculae completely coated with osteoid The thought behind this question of course is whether the osteoid under certain conditions does not insulate the bone salt from the body fluids

Park The experimental animals had full development of rachitic osteoid the protected controls did not

Dr McCollum Dr Shipley and I did an experiment Dr McCance which was rather interesting although perhaps not bearing directly on the question you have raised Some rats were placed on a low calcium diet and the females were allowed to become pregnant and give birth to young The young were reared on the low calcium diet and then they were allowed to become pregnant and give birth to a second generation of young In the first generation the animals on the low calcium diet showed very little change in the skeleton In the second generation they showed some evidences of low calcium rickets In the third generation the ribs were so filled with fractures that the bones were just dotted with them from one end to the other

Kramer I can add to that because I did the calcium determinations on those animals A progressive decrease in the calcium level of the blood was seen from generation to generation Another interesting point is that in rats that had been rendered rachitic with a high calcium low phosphorus diet a few days of starvation produced a tremendous rise in the serum inorganic phosphorus and initiated very striking healing

McCance Dr Stearns has produced some evidence that the poor absorbers in these native populations may have been bred out and this is experimental evidence from animals pointing to the same thing Would we agree on that?

Reifenstein Dr Park I would like to ask about the parathyroid glands

[185] This statement by Dr Park has been revised as the result of the discovery of Dr Harrison's unpublished thesis At the meeting Dr Park reported Dr Harrison's experiments from memory and made the error of thinking that Dr Harrison had used the Steenbock rachitogenic diet with the phosphorus content made up so that the calcium phosphorus ratio was optimal

in those animals Was there a decided difference between the gland of the two groups?

Parl I cannot say anything about that I do not know

Reifenstein I am raising the question as to whether there is a difference in the ability of the parathyroid glands to compensate for a low level of calcium in various individuals In the patient that Dr McCance described with the very low serum inorganic phosphorus level one wonders if there was a considerable degree of secondary parathyroid hyperplasia with increased hormone production which was helping to keep the serum calcium level from falling I would be interested in knowing the serum phosphorus level in these other vegetarians Perhaps they were successful in adapting to low intakes of calcium because they were particularly adept in developing a compensatory parathyroid hyperplasia

McCance I will try to find out

Armstrong What is a poor absorber of calcium? You say that a person is a good absorber of calcium if he keeps his skeleton in that situation which you regard as normal Actually Dr McCance your patient was a subject who overdid supporting blood calcium through mobilization of the calcium of the skeleton Can you be sure that she was really not absorbing calcium to the normal degree True enough she did have a low calcium intake her supply of calcium was low but was she absorbing that more poorly than would some other individual on the same diet?

McCance I would think she was Otherwise the other vegetarians would have decalcified themselves

Bartter When she was on 6 0 mg of calcium every bit of it came out in the feces Is not that enough to make her a poor absorber

Stearns Her urinary calcium was very low as low as that of most babies 10 mg

Armstrong This was after her calcium had been depleted

Follis Dr McCance how much does vitamin D change your own absorption?

McCance Not very much But I have a very marked seasonal variation in my calcium absorption[1] I followed it over a period of two or three years and it is very much better in Summer and Autumn than it is in

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[1] McCance R A and Widdowson E M Seasonal and Annual Changes in the Calcium Metabolism of Men, *J Physiol* 10 4 (1943)

animals to maintain the concentrations of calcium and phosphorus at normal levels during fasting[185]

Albright Dr Park, were the bones different in the two groups of animals you have just described?

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Albright I was wondering whether in those cases of rickets where the blood values are less stable one finds the trabeculae completely coated with osteoid. The thought behind this question, of course, is whether the osteoid under certain conditions does not insulate the bone salts from the body fluids.

Park The experimental animals had full development of rachitic osteoid; the protected controls did not.

Dr McCollum, Dr Shipley, and I did an experiment, Dr McCance, which was rather interesting, although perhaps not bearing directly on the question you have raised. Some rats were placed on a low calcium diet, and the females were allowed to become pregnant and give birth to young. The young were reared on the low calcium diet, and then they were allowed to become pregnant and give birth to a second generation of young. In the first generation the animals on the low calcium diet showed very little change in the skeleton. In the second generation they showed some evidences of low calcium rickets. In the third generation the ribs were so filled with fractures that the bones were just dotted with them from one end to the other.

Kramer I can add to that because I did the calcium determinations on those animals. A progressive decrease in the calcium level of the blood was seen from generation to generation. Another interesting point is that in rats that had been rendered rachitic with a high calcium low phosphorus diet, a few days of starvation produced a tremendous rise in the serum inorganic phosphorus and initiated very striking healing.

McCance Dr Stearns has produced some evidence that the poor absorbers in these native populations may have been bred out, and this is experimental evidence from animals pointing to the same thing. Would we agree on that?

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Park: I cannot say anything about that. I do not know.

Robinson: I am raising the question as to whether there is a difference in the ability of the parathyroid glands to compensate for a low level of calcium in various individuals. In the patient that Dr. McCance described with the very low serum inorganic phosphorus level, one wonders if there was a considerable degree of secondary parathyroid hyperplasia with increased hormone production which was helping to keep the serum calcium level from falling. I would be interested in knowing the serum phosphorus level in these other vegetarians. Perhaps they were successful in adapting to low intakes of calcium because they were particularly adept in developing a compensatory parathyroid hyperplasia.

McCance: I will try to find out.

Armstrong: What is a poor absorber of calcium? You say that a person is a good absorber of calcium if he keeps his skeleton in that situation which you regard as normal. Actually, Dr. McCance, your patient was a subject who overdid supporting blood calcium through mobilization of the calcium of the skeleton. Can you be sure that she was really not absorbing calcium to the normal degree? True enough, she did have a low calcium intake, her supply of calcium was low, but was she absorbing that more poorly than would some other individual on the same diet?

McCance: I would think she was. Otherwise the other vegetarians would have decalcified themselves.

Park: When she was on 650 mg. of calcium every bit of it came out in the feces. Is not that enough to make her a poor absorber?

Stearns: Her urinary calcium was very low, as low as that of most babies, 10 mg.

Armstrong: This was after her calcium had been depleted.

Follis: Dr. McCance, how much does vitamin D change your own absorption?

McCance: Not very much. But I have a very marked seasonal variation in my calcium absorption.¹ I followed it over a period of two or three years, and it is very much better in Summer and Autumn than it is in

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¹ McCance, R. A., and Widdowson, E. M.: Seasonal and Annual Changes in the Calcium Metabolism of Man. *J. Physiol.* 102:4 (1943).

Winter and Spring. But I cannot put my Winter absorption up to Summer absorption levels by taking 2000 units of vitamin D a day.

Harrison: You may be vitamin D resistant.

McCance: I may be. I may be all these things, but for all that I am still able to lead a fairly active healthy life. For instance, I cycled 120 miles on one day this Spring. I still regard myself as being within the limits of normality.

Armstrong: Which vitamin D have you used?

McCance: I used calciferol for that experiment.

Armstrong: There is a difference between the sunshine vitamin D and calciferol, at least when one compares the chick and the rat, isn't there?

McCance: Am I the rat or the chicken?

Armstrong: I suggest that you are the chicken.

McCance: The chicken? Splendid! [Laughter]

Follis: Is there any evidence that there is any relationship between the people who are poor absorbers and those who may be a little more resistant to vitamin D, or don't you have data on this point?

McCance: I do not quite understand what you mean.

Follis: I mean, you are a poor absorber, and you say that you do not respond as well as others to vitamin D.

McCance: None of our subjects had their calcium absorptions changed appreciably by 2000 units a day.[13]

Kramer: How about the retention? Was there a difference in retention? After all, that is the important factor in normal bone growth.

McCance: No. We reckoned that vitamin D made no appreciable difference to our calcium retention.

Kramer: I would like to ask Dr. Stearns what is the usual amount of calcium and phosphorus that must be retained daily by a child or adult to insure bone growth?

Stearns: That depends on the person to whom you are speaking, Dr. Kramer. Everyone has a different idea.

Kramer: You have done a lot of metabolic work on this point so I thought I would ask you about it.

COMMENTS ON THE INTAKE OF CALCIUM AND PHOSPHORUS REQUIRED FOR BONE GROWTH

GENEVIEVE STEARNS

From the Department of Pediatrics, State University of Iowa, Iowa City, Iowa

Armstrong: Dr. Stearns, do you wish to discuss this point?

Stearns: The amount of calcium and phosphorus that must be retained daily to insure bone growth will vary, I think, with the age of the child and the rate of growth that is normal and customary for that age. So that on a day-to-day requirement, calcium retention is lowest at about three years of age and highest in infancy and in the rapid pre-puberal period of growth. But if you follow children through the entire growth period, as we have tried to do,[1,2] you find that they will tend to increase the calcium retention according to the growth in weight rather than the growth in length, which means that they store calcium for a couple of years, if they are permitted to, before the rapid pre-puberal spurt of growth. If their intake does not permit it, they do the best they can. But apparently, regardless of the particular intake they are given, the amount that they retain will follow the type of curve for rate of growth consistent for their age and weight; that is, if you give children at different ages 0.2 gm. of calcium, you will get a curve of retention which tends to parallel roughly what you get if you give them each one gram of calcium daily. With the gram of calcium, the whole curve rises. There are many factors that enter into it besides that. Phosphorus retention must cover both calcium and nitrogen retention.

Kramer: I was thinking about what the limits are in any one age group.

Stearns: Up to 10 or 11 years, a child who gets three glasses of milk or three quarters of a liter a day will retain an ample amount of calcium for all the skeletal needs. If he is given more, he retains more. In babies and up to about the end of the first year, the more you give, the higher the percentage they will retain. Older children fed cow's milk apparently reach an upper limit of retention.

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[1] Stearns, G.: The Significance of the Retention Ratio of Calcium: Phosphorus in Infants and in Children. *Am. J. Dis. Child.* 42: 749-759 (1931).

[2] Stearns, G., and Moore, D. L. R.: Growth in Height and Weight and Retention of Nitrogen, Calcium and Phosphorus During Recovery from Severe Malnutrition. *Am. J. Dis. Child.* 42: 774-780 (1931).

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McCance I used calciferol for that experiment

Armstrong There is a difference between the sunshine vitamin D and calciferol at least when one compares the chick and the rat isn t there?

McCance Am I the rat or the chicken?

Armstrong I suggest that you are the chicken

McCance The chicken? Splendid! [Laughter]

Follis Is here any evidence that there is any relationship between the people who are poor absorbers and those who may be a little more resistant to vitamin D or don t you have data on this point?

McCance I do not quite understand what you mean

Follis I mean you are a poor absorber and you say that you do not respond as well as others to vitamin D

McCance None of our subjects had their calcium absorptions changed appreciably by 2000 units a day [1]

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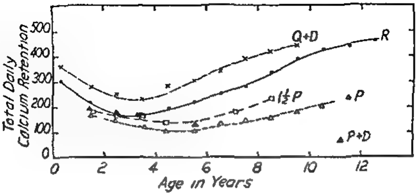

Fig. 51. The Mean Daily Calcium Retention of Children of Various Ages in relation to the Daily Intake of Milk and of Vitamin D

Curve R (the solid circles) represents the desired daily calcium retention estimated from the calcium content of a well calcified adult skeleton and the normal rate of growth for each given year. *Curve P (the open triangles)* represents the daily calcium retention when one pint of milk was included in the daily diet. *Curve P + D (the solid triangles)* represents the daily calcium retention when one pint of milk and 300 to 400 International Units of vitamin D were included in the daily diet. *Curve 1½ P (the open squares)* represents the daily calcium retention when one and a half pints of milk were included in the daily diet, and *curve Q + D (the crosses)* represents the daily calcium retention when one quart of milk and 300 to 400 International Units of vitamin D were included in the daily diet.

vitamin D daily *they do retain very much more*. These children were healthy at the time of the study. Of course children lose calcium quite markedly during intercurrent infections and that is an age when infections are common. But the three year old fed a low intake retained less than the one year old or the twelve year old and in the same way the three year old fed the quart of milk never retained as much as the children with the more rapid rate of growth, so that the actual rate of growth desirable at the time does apparently have something to do with it.

In Figure 52 the data are calculated in milligrams per kilogram against the same theoretical curve. This group received between 25 and 50 mg of calcium a day, which is a fairly wide spread. There is a definite rise with age in the amount retained in milligrams per kilogram daily and the peak comes at 8 to 10 years of age which is before the age when the children begin to grow rapidly in height. I have interpreted this finding as more evidence that there is a period of storage before they begin the rapid pre-puberal growth. This is like the old Stratz theory that you have periods of "filling" and periods of "stretching" and the calcium data tend to sup-

Urist Can children deposit bone salt continuously in unlimited quantities during the period of growth?

Stearns No A three year old given a quart of milk will not retain as much as a six month old or as a twelve year old

McCance These experiments of yours are very well known, and they are of course fundamental to all our knowledge of calcium metabolism I would like to ask one question Where do you picture the calcium is being stored? It must be in the bones Therefore when the bones begin to grow rapidly there must be a redistribution of calcium in that bone Is that how you picture the process taking place?

Stearns I think so Dr McCance Once we had the opportunity to study a three year old child who had been very badly undernourished He weighed 17 pounds which is the average weight for six months and he was 17 centimeters under height for three years of age We found that as soon as he began to retain he retained as much calcium per kilogram as a young baby during the period of rapid growth I think it was about 60 mg per kilogram which is very high for a three year old We were measuring his length at weekly intervals He did not grow in length at all until after he had exhibited that high daily retention for a period of six weeks and then he started to grow in length and grew 7 centimeters in the next six weeks Then he stopped growing in length for another six or eight weeks In the meantime he kept retaining large amounts of calcium He made up his weight loss in three months but it took him three years to make up his height deficiency he did make it up but always by alternate periods of apparent saturation and then growth saturation and then growth

Follis This indicate only that there are two different phases—growth of cartilage and growth of bone In other words chondrogenic and osteogenic activity You can dissociate the two very easily

Stearns In Figure 51 are shown the data on the retentions we obtained with children of these ages given a pint and a half of milk We almost duplicated our theoretical retention curve which is a little higher than Dr Mitchell s I think because we used as our normal calcium content of bone the average calcium content of the bone of animals fed on known diets rather than using the calcium content of the bone of either the German suicide patient or Dr Mitchell s elderly man who died of heart failure[119] You see if the children are fed a quart of milk and 300 to 400 units of

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[119] Mitchell H H Hamilton T S Steggerda F R and Bean H W The Chemical Composition of the Adult Human Body and Its Bearing on the Biochemistry of Growth *J Biol Chem* 158 625 (1945)

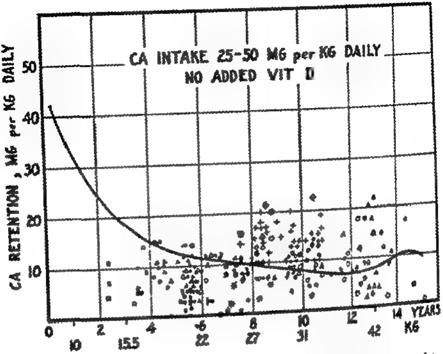

Fig 52 The Calcium Retention of Children in relation to Age and to the Theoretical Calcium Retention when No Vitamin D Is Added

The retention data are expressed in mg per kg per day with a calcium intake range of 25 to 50 mg per kg per day. *The solid line* represents the theoretical calcium retention. It will be noted that the calcium retention tends to rise at about 8 years of age, well before the pre-puberal growth spurt. These data suggest that calcium storage may be a necessary prelude to increased bone growth.

port this concept. At 10 years they will retain from the same range of per kg intake almost double the calcium that children under 8 years will retain from the same per kg intake. The period of rapid skeletal growth comes along at about 12 years of age.

Conference Discussion

Reifenstein: I would like to ask about one point. Does the amount of calcified bone present in an adult, say 40 years of age, have a relationship to the amount of calcium that he requires to stay in equilibrium? I can think of an adult who is five feet tall and has a relatively small bone structure and I can think of another one who is 6'6" with a large bone structure; both of them are normal in every respect but one has much more calcified mass than the other. Is the amount of calcium required to keep

he be one in equilibrium greater than that needed by the small?

McCance I could not tell you It is possible But size is not the explanation for all the differences between poor and good

Reifenstein That is what I believe also

McCance I can say that with confidence

Stearns It was the big men who went to pieces in the Japanese concentration camps and the little men who came through best I am told *The diet was very low in calcium and very low in many other things* but it was the big framed men who suffered most

McCance Yes but did they not suffer from general undernutrition and B vitamin deficiencies rather than specific calcium deficiency?

Stearns But are not their requirements in proportion to their size generally When we were studying urinary calcium excretion we found that the men who were 6 4 and big built proportionately never could get down to the urinary excretion with a low intake that the very small the 5 3 men could The skeletal size does have something to do with it

Sobel When we come to the fat soluble vitamins especially vitamin A which has been studied simply because there is a method available it is well known that there are poor absorbers of vitamin A In the poor absorbing group you have the celiac syndrome and the so called nontropical sprue Nontropical sprue has been studied considerably by a number of workers among them Spies and Darby and if one does a vitamin A absorption test before any dietary treatment by the test there seems to be extremely poor absorption If one gives such patients vitamin B complex (which is Spies's approach) or folic acid (which is Darby's approach) the vitamin A absorption is improved

After I saw these data in the literature I tried the procedure on a few of our patients who fell into two categories One was the patient with the so called celiac syndrome who was arbitrarily given multivitamin plus folic acid every day and the vitamin A absorption curve tested before and after such a course of treatment altered considerably Then we had one particular patient who was studied in many institutions including the Presbyterian Hospital because he had the lowest cholesterol known in an adult as far as we could discern approximately 50 mg per 100 cc When we saw him—he came from a chronic disease hospital—he could not move his legs and could just about move his hand Well after we had studied

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Sobel A E The Problem of the Absorption and Transportation of Fat Soluble Vitamins *Vitamins and Hormones* 10 4 (1952)

Butler Is somebody going to discuss the effect of cortisone on calcium absorption?

Armstrong Would you like to discuss this point Dr Butler?

Butler No

McCance Are you looking at me with the idea that I am going to discuss it?—I couldn t

Butler No but I just wondered if the difference in calcium absorption could be related to that factor Dr Howard tells me he improved the calcium absorption in the patient with sprue by giving cortisone

Howard Yes that is true but sprue is a very abnormal condition which somehow or other you seem to correct when you give cortisone We did not do calcium balance experiments on any of the rheumatoid arthritics or asthmatics treated with ACTH or cortisone We only measured the urine and the blood and could find no change in them But there are good experiments in the literature with large doses of cortisone and most of them give the impression that at first there is really no essential change in absorption as judged from the fecal or the urinary excretion and that later there is perhaps a slight tendency to a negative balance certainly not very marked as indicated by slight increases in the stool and perhaps in the urine calcium Certainly it does not give any one the impression that if one took 200 or 300 mg of cortisone a day over a period of a couple of years one would end up with a very rare skeleton

Butler It does not give you that impression?

Howard Not to me no The best experimental data that I have seen were three month experiments done by some Swedish investigators and the change in calcium balance certainly was not very impressive

Albright In Cushing s syndrome which is due to the overproduction of cortisone like steroids osteoporosis of the spine is a very common finding

Howard That is a secret of Cushing s syndrome that I would love to know—how the patients with it rarefy that bone

Henneman We know that osteoporosis certainly does develop in Cushing s disease medicamentosa

Howard Have you ever seen it?

Henneman Yes I have seen three cases recently

Howard Three people who have had rarefied bones induced during cortisone administration?

him very intensively he turned out to be a nonabsorber of fats including vitamin A and his absorption even for cholesterol was extremely low We made a tentative working hypothesis that he might be the result of a prolonged fat soluble vitamin deficiency which went back to his childhood and that as a result many secondary changes had taken place To test this postulate the patient was given large amounts of multivitamins in aqueous dispersion plus folic acid for several weeks and then a second vitamin A absorption test was done this time the vitamin A absorption had improved considerably The test was repeated a few weeks later after he had continued the same therapy still longer and the vitamin A absorption had continued to improve in fact the patient seemed to us to have begun to use some of the muscles that he had been unable to use before but the clinician in charge said that this apparent change was not definite Unfortunately the patient went back to the chronic disease hospital and we lost track of him

I would like to add one more fact Many years ago before we knew what vitamin B was and we knew only about the vitamin B complex in order to satisfy Dr Kramer and to attempt to produce incurable rickets we destroyed the vitamin B complex in the diet and allowed rickets to develop Such animals did not grow at all in fact they lost a little weight But they did develop rickets that was quite distinct I still have the slides When we gave such animals fairly large amounts of vitamin D which would cure the rickets in the control animals very elegantly in a period of 9 days there was some healing in the B complex deficient group but it was very mild But since there was some healing I did not say much about it and never published it because we did not establish rickets in which there really was no healing

It seems to me that by dietary means one can alter the absorption at least of vitamin A and probably of vitamin D and to some measure this change seems to be reversible If the folic acid experiment and if Spies's observations mean anything it is possible that the kind of patient that Dr McCance observed had other deficiencies of prolonged standing which in turn altered the intestinal mucosa as well as the intestinal secretions in such a way that calcium absorption was poor merely because there was an altered intestine and an altered intestinal secretion I am referring particularly to the bile acid and the pancreatic juices which might be influenced by a nutritional defect Is it possible that if you had intensively treated the patient with amino acid hydrolysates *plus* the vitamin B complex *plus* folic acid you might in due time have reversed the picture and restored her to the category of a person with a normal absorption of calcium?

McCance It is possible

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Howard Three people who have had rarefied bones induced during cortisone administration?

him very intensively he turned out to be a nonabsorber of fats including vitamin A and his absorption even for cholesterol was extremely low. We made a tentative working hypothesis that he might be the result of a prolonged fat soluble vitamin deficiency which went back to his childhood and that as a result many secondary changes had taken place. To test this postulate the patient was given large amounts of multivitamins in aqueous dispersion plus folic acid for several weeks and then a second vitamin A absorption test was done this time the vitamin A absorption had improved considerably. The test was repeated a few weeks later after he had continued the same therapy still longer and the vitamin A absorption had continued to improve in fact the patient seemed to us to have begun to use some of the muscles that he had been unable to use before but the clinician in charge said that this apparent change was not definite. Unfortunately the patient went back to the chronic disease hospital and we lost track of him.

I would like to add one more fact. Many years ago before we knew what vitamin B was and we knew only about the vitamin B complex in order to satisfy Dr Kramer and to attempt to produce incurable rickets we destroyed the vitamin B complex in the diet and allowed rickets to develop. Such animals did not grow at all in fact they lost a little weight. But they did develop rickets that was quite distinct. I still have the slides. When we gave such animals fairly large amounts of vitamin D which would cure the rickets in the control animals very elegantly in a period of 9 days there was some healing in the B complex deficient group but it was very mild. But since there was some healing I did not say much about it and never published it because we did not establish rickets in which there really was no healing.

It seems to me that by dietary means one can alter the absorption at least of vitamin A and probably of vitamin D and to some measure this change seems to be reversible. If the folic acid experiment and if Spies's observations mean anything it is possible that the kind of patient that Dr McCance observed had other deficiencies of prolonged standing which in turn altered the intestinal mucosa as well as the intestinal secretions in such a way that calcium absorption was poor merely because there was an altered intestine and an altered intestinal secretion. I am referring particularly to the bile acid and the pancreatic juices which might be influenced by a nutritional defect. Is it possible that if you had intensively treated the patient with amino acid hydrolysates *plus* the vitamin B complex *plus* folic acid you might in due time have reversed the picture and restored her to the category of a person with a normal absorption of calcium?

McCance It is possible

Butler Is somebody going to discuss the effect of cortisone on calcium absorption?

Armstrong Would you like to discuss this point Dr Butler?

Butler No

McCance Are you looking at me with the idea that I am going to discuss it?—I couldn t

Butler No but I just wondered if the difference in calcium absorption could be related to that factor Dr Howard tells me he improved the calcium absorption in the patient with sprue by giving cortisone

Howard Yes that is true but sprue is a very abnormal condition which somehow or other you seem to correct when you give cortisone We did not do calcium balance experiments on any of the rheumatoid arthritics or asthmatics treated with ACTH or cortisone We only measured the urine and the blood and could find no change in them But there are good experiments in the literature with large doses of cortisone and most of them give the impression that at first there is really no essential change in absorption as judged from the fecal or the urinary excretion and that later there is perhaps a slight tendency to a negative balance certainly not very marked as indicated by slight increases in the stool and perhaps in the urine calcium Certainly it does not give any one the impression that if one took 200 or 300 mg of cortisone a day over a period of a couple of years one would end up with a very rare skeleton

Butler It does not give you that impression?

Howard Not to me no The best experimental data that I have seen were three month experiments done by some Swedish investigators and the change in calcium balance certainly was not very impressive

Albright In Cushing s syndrome which is due to the overproduction of cortisone like steroids osteoporosis of the spine is a very common finding

Howard That is a secret of Cushing s syndrome that I would love to know—how the patients with it rarefy that bone

Henneman We know that osteoporosis certainly does develop in Cushing s disease medicamentosa

Howard Have you ever seen it?

Henneman Yes I have seen three cases recently

Howard Three people who have had rarefied bones induced during cortisone administration?

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Howard Have you ever seen it?

Henneman Yes I have seen three cases recently

Howard Three people who have had rarefied bones induced dur cortisone administration?

Henneman Yes

Urist Yes I too have seen osteoporosis induced by prolonged treatment with cortisone

Howard You knew what the bones looked like before and afterward?

Urist Only that they have osteoporosis in x ray examination after treatment with cortisone given as replacement therapy following adrenalectomy or for malignant tumors or rheumatoid arthritis

Howard Which they did not have before

Urist There was not always an occasion to make a special pretreatment x ray examination The bone changes produced by cortisone are however progressive and quite definite

Howard I have seen that too but nearly all rheumatoid arthritics have very rare bones

Henneman The patients we have seen recently present clinical histories indicative of relatively normal bones before treatment Following prolonged and intensive treatment with ACTH or cortisone they have developed back pain lost height and developed bone rarefaction and fractures by x ray Extreme osteoporosis has developed in one year of intensive induced hyperadrenocorticism

Harrison What is the mechanism in those patients as determined by balance study? Is it excessive loss in the urine or poor absorption of calcium in your induced Cushing s syndrome?

Henneman The administration of large doses of cortisone to patient on balance studies produces a rise in urinary cal tion without a change in fecal calcium excretion or blood calciu e believe this reflect inhibition of osteogenesis (anti anabolis e destructio continues at an unchanged rate

Harrison I stion because Pitts the effe of cortisone on t reabsorption of p He found that cortis the maximum rption phosphate The e ne on bone ma of lo phosphorus and ca m phosphorus cortisone does decr bably more

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[1]Roberts K and Pitts rt search Conference on Calc ls

the pretreatment concentrations of serum phosphorus are higher in children than in adults

Henneman Am I correct that in the ACTH studies that you did Dr Bartter there was no very constant effect of ACTH on serum phosphorus?

Bartter The serum phosphorus tended to fall There was a fairly consistent increase of urinary calcium but in retrospect it appeared as though that might have been due to contaminating pitressin

Shorr May I speak on this point? We have been studying a patient with rheumatoid arthritis who has been receiving 87 5 mg of cortisone orally for 2 years and who has developed profound osteoporosis By increasing her intake of calcium up to 3 2 gm per day of which 1 5 gm are supplied in the food her significantly negative calcium balance is not corrected These high calcium losses are not accounted for by her renal excretion of calcium it occurs chiefly in the stools It is also of interest that although she is in strongly negative calcium balance she is in significantly positive nitrogen balance

Urist Why is the osteoporosis more pronounced in the spine? It is reasonable to suppose that all of the bones of the body will show some demineralization but the rarefaction of the spine is out of all proportion to that of the rest of the skeleton

Shorr I cannot offer any reasons for the exaggerated osteoporosis which is so commonly seen in the spine but I feel sure that the process of demineralization is general in most cases What I should like to comment on is the extent to which our actual metabolic observations on the relation of nitrogen to calcium deposition permit us to use overall balance studies for this purpose With this patient our first concern was to see whether we could overcome her negative calcium balance by nutritional means alone and as I have said we are unable to do this by raising her calcium intake We have attempted also to promote nitrogen storage by raising her protein intake without success

Reifenstein May I ask what happens to the alkaline phosphatase in the induced rarefaction of bone which occurs when you give cortisone? Does the alkaline phosphatase stay the same or does it rise?

Shorr It remains within the normal range

Reifenstein It is not elevated whereas in metabolic bone conditions in which calcium is being resorbed from bone we tend to have a rise in the alkaline phosphatase level presumably due to an increase in compensatory bone formation

Urist Dr Shorr was your patient adrenalectomized?

Shorr No this is a case of rheumatoid arthritis

Bartter Dr Shorr is it so difficult to conceive of a negative bone matrix nitrogen balance with a positive overall nitrogen balance?

Shorr It is perfectly conceivable but improvable by the conventional balance studies Let me restate my present opinion The necessity for the presence of a protein matrix as a prerequisite for calcium deposition has been clearly established for almost 70 years The recent efforts to buttress this observation are based on conventional balance studies utilizing intake and output of calcium nitrogen and phosphorus When such simple indices are used one finds significant discrepancies between nitrogen storage and calcium deposition one may be positive and the other negative or *vice versa* For example in this specific case of cortisone osteoporosis the patient is in positive nitrogen balance of almost 2 grams a day and in a negative calcium balance of well over 300 mg a day Hence such balance studies cannot be used as support for this concept There is no way to insure from the balance study that protein is retained in any specific tissue when the balance is positive or lost in any specific tissue when the balance is negative

Robinson What was the alkaline phosphatase at that time? Was it done?

Shorr Yes The alkaline phosphatase remained within the normal range We do not know her pre cortisone balance as she was referred to us for study when her osteoporosis was well established after a little more than about a year and a half of continued cortisone administration

Shorr The alkaline phosphatase level is not elevated in most cases of osteoporosis

Reifenstein Thus the failure of the alkaline phosphatase level to rise is against the suggestion of Dr Harrison that the mechanism of cortisone on bone is due entirely to increased loss of calcium in the urine from an effect on the kidney Such a mechanism would favor bone resorption and a compensatory rise in alkaline phosphatase in contrast to what we actually find

Butler I would like to ask again with all this evidence that cortisone tends to cause a negative calcium balance is anybody going to offer an explanation of why it improves the calcium balance in a patient with sprue?

Bassett It improves fat absorption in sprue presumably That might have some bearing on it

Butler Why does cortisone improve fat absorption?

Bassett Nobody knows

Henneman Sprue raises a separate problem for it is primarily a disease of intestinal function Cortisone somehow ameliorates the intestinal abnormality in sprue and restores calcium absorption (or excretion) toward normal In persons with normal intestinal function cortisone increases urinary calcium excretion and this cannot be accounted for by increased calcium absorption from the intestines

Shorr I should have to question that because as we increased her intake our patient continued to lose more calcium in the stool not in the urine

Henneman You mean that she loses a larger per cent of her calcium intake?

Shorr As we have been raising her calcium intake she has been losing more and more via the intestinal tract

Henneman Has cortisone increased urinary calcium excretion in this patient?

Shorr Very minimally Practically all the loss was in the stool when her calcium intake was raised above 800 mg per day

Armstrong She has a very high intake

Shorr It is now 3 2 grams of calcium per day of which 1 5 grams is dietary

Henneman As far as I know if the calcium intake is kept constant cortisone produces a rise in urinary calcium excretion and no change in fecal calcium excretion

Urist Dr Shorr was your patient adrenalectomized?

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DISEASES PARTICULARLY OF BONE, ASSOCIATED WITH DERANGEMENTS OF CALCIUM AND PHOSPHORUS METABOLISM

RICHARD H FOLLIS JR

From the Department of Pathology The Johns Hopkins University School of Medicine and The Johns Hopkins Hospital Baltimore Maryland

Armstrong We now come to the discussion of the topics which have been the real reason for all that has gone before in the ■ Conferences because we desire and need practical treatment for diseases of the skeleton There is a second reason which justifies consideration of bone diseases which is even more important because through a study of abnormalities of the skeleton we learn a good deal about the normal processes I am reminded here of the often quoted statement of Osler that as clinicians we examine the experiments of nature I am using we here in the general sense I do not think that this description applies to me It does apply to our good friend Dr Shorr Dr Robinson and several others As far as I am concerned I am like Dr Sobel I am a rat doctor [Laughter]

Dr Follis will introduce the subject of bone diseases

Introduction

Follis : I am not going to take up all bone diseases but only those in which there are disturbances in calcium and phosphorus metabolism either as evidenced by changes in their humoral concentrations or by changes in their excretion

Figure 53 is just a review We must keep in mind certain important factors in the development of the skeleton in the growing skeleton as well as that of the adult For growth there has to be proliferation and maturation of the epiphyseal cartilage cell In the matrix between the hypertrophic cell inorganic elements namely calcium and phosphorus are deposited Then the cartilage cells imbedded in this calcified matrix are destroyed or die This leaves a framework of impregnated organic cartilage matrix and on this scaffolding as it were osteoblasts in some very obscure way promote the formation of osteoid which i the organic matrix of bone In such osteoid as you well know inorganic material then depo it In order that bone may not be excessively dense as soon as it i formed a large part of it is immediately destroyed

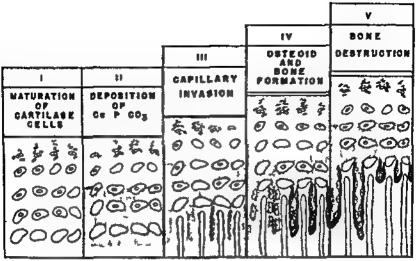

Fig 53 A Schematic Breakdown of the Events Which Occur in the Growth of the Skeleton

A Classification of Microscopic Bone Disease

We have classified bone diseases for you on other occasions We can divide bone diseases with respect to normal formation First there are those characterized by disturbances in the growth of the cartilage second there may be abnormalities in the balance between the destruction and the formation or *vice versa* and third are those diseases which are associated with disturbances in the deposition of inorganic materials in the cartilage matrix and in the bone matrix

Table XXV shows the possibilities that one can see microscopically I placed microscopic in the heading of this table so that it is not necessary to worry about the difficulties which the roentgenologist has in determining the type of bone disease with which he is dealing There may be bones with decreased density which may arise either as a result of decreased production of bone matrix or of excessive destruction We will come back to this condition in a moment There may be excessive bone formation or so called osteosclerosis in which the amount of bone is increased There may be excessive destruction and by that I mean microscopic evidence of destruction over and above the normal amount We will also come back to that And then there may be disturbances in the deposition of inorganic

TABLE XXV

Microscopic Bone Disease

A	OSTEOPOROSIS	Decreased Production and Increased Destruction
B	OSTEOSCLEROSIS	Increased Production and Decreased Destruction
C	OSTEITIS FIBROSA	Excessive Destruction
D	RICKETS OSTEOMALACIA	Defect in Deposition of Inorganic Elements

materials in the organic matrix and that of course we call rickets in the growing child and osteomalacia in the adult It is primarily the last two conditions (Table XXV C and D) that we will discuss in this presentation In these disturbances in calcium and phosphorus metabolism manifest themselves and may lead to disease of bone

The Evolution of Rickets in the Growing Child

You are familiar I am certain with the changes which one sees in rickets in the growing child Figure 54 A and B show the earliest changes that one can detect these illustrations are taken from a series that Dr Park and I have been studying for the last few years [19] You see areas between the cartilage rows where black stained material (which represents inorganic salt) has failed to deposit As time goes on of course the defective de position becomes greater and greater And so one ends up with the picture of excessive or severe chronic rickets This of course is the stage that is familiar to all of you who have used the rat in studies of calcification and so forth

In the shaft one also finds evidence of faulty deposition of inorganic materials (Figure 55) In the trabeculae one observes lighter staining areas that represent organic matrix which has not been impregnated with inorganic materials I might point out also that this coating is rather marked on one side and not so marked on the other I would like to say further more that I do not think that this coating forms a mantle or a barrier which interferes with the transport of calcium back and forth

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Follis R H Jr Park E A and Jackson D The Prevalence of Rickets at Autopsy During the First Two Years of Age *Bull Johns Hopkins Hosp* 91 480 (1952)

Bartter Why not? [33]

Follis Well it is not a uniform covering in the first place

Bartter But in severe cases it might be

Follis We have no evidence actually that it would act as an insulator

All right ' Have you any evidence that it would not?

Follis No not direct Do you think so Dr Park?

Park I do not see how What you know is that when it is present there also are immense amounts of bony surface which do not have it

Engel Why would not that coating interact with calcium ions like any other tissue anywhere else? There need not be apatite present but you could still have bound calcium and ionic calcium in that layer which would be a function of the colloid there

Shorr We have evidence also do we not in the *in vitro* studies of the deep penetration of calcium and phosphorus into the rachitic cartilage?

Follis That is an important point I think Dr Shorr in other words the inorganic materials in *in vitro* calcification do not deposit on the surface As Shipley and his colleagues [9] pointed out twenty five years or so ago these substances are deposited down deep in the cartilage

Shorr Yes that is right

Follis What the reason is I do not know

As the child ages the cartilage begins to decrease in activity Then one does not detect rickets at the cartilage shaft junction In Figure 56 of a five year old child all one can find is osteoid in the shaft Therefore the consideration that was raised previously at this conference is important that is that growth is necessary for the development of rachitic changes particularly those in the cartilage

The Pathogenesis of Rickets and Osteomalacia

Now what are some of the possibilities which may lead to changes in the

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Bartter (comment submitted after the Conference) If it be agreed (as Dr Follis appears to agree) that bone decalcification does not take place ... without bone destruction that is removal of matrix then evidence that calcification can take place through osteoid seams ... beside the point The osteoid needs only protect matrix from being removed not to block passages of ... n

Shipley P G Kramer B and Howland J Studies upon Calcification *In Vitro* *Biochem J* 20 379 (1926)

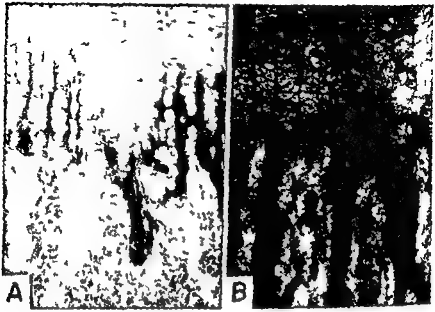

Fig. 54. Early Rickets at the Cartilage Shaft Junction of Human Ribs.

A—An area of defective deposition of inorganic materials (no dark staining areas). *B*—A more acute and complete absence of deposition of inorganic materials.[194]

humoral concentrations? As you recall, Dr. Kramer and Dr. Howland[195] showed thirty years ago the importance of the humoral concentrations, the calcium and phosphorus product of the serum concentrations, in relation to whether or not one could expect to find rickets in growing children. In Table XXVI we have a background of the various possibilities. I am certain I have omitted some, but I think we have covered most of them.

MATRIX PRODUCTION IN EXCESS OF MINERAL DEPOSITION

In the first place, there may be a disturbance in the production of matrix and the deposition of inorganic material in that matrix. In other words, given normal humoral concentrations of calcium and phosphorus, can matrix production outstep the deposition of inorganic material in it? I think this probably happens. We recognize it in rapidly growing children and in rapidly growing rats, where we find no demonstrable rickets at the cartilage shaft junction, but where we do find, as we will see in a moment

[195] Howland, J., and Kramer, B.: Factors Concerned in the Calcification of Bone. *Trans. Am. Pediat. Soc.* 34: 204 (1922).

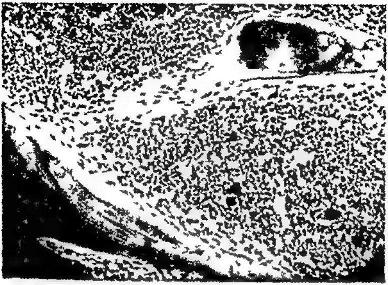

Fig 55 Osteoid in the Shaft of Bone in Human Rickets

Note the relative uniformity of the osteoid coating (light staining material) on the bone (which stains more darkly)

[Reproduced by permission from Follis R H Jr *The Pathology of Nutritional Disease* Charles C Thomas Publisher Springfield Illinois p 111 (1948)]

osteoid in the shaft One observes probably the same situation in healing fractures or in healing scurvy in which there is an excessive production of bone matrix and as Dr Albright and Dr Reifenstein have pointed out[98] one sees the same situation in healing osteitis fibrosa after the removal of a parathyroid tumor

Figure 57 illustrates the cartilage shaft junction from a premature rapidly growing child which we probably would say shows a normal deposition of inorganic materials at the cartilage shaft junction When you get down into the shaft you find borders of osteoid Whether or not you can interpret this as excessive that is whether or not you can conclude that this individual had rickets is an academic point I believe that Dr Park and I in the premature at this age would say that this is physiological osteoid I think Dr McLean and Dr Bloom[9] have used the term "physiological osteoid

[98] Albright F and Reifenstein E C Jr *The Parathyroid Glands and Metabolic Bone Disease Selected Subjects* Williams and Wilkins Co Baltimore p 104 (1948)

Fig 56 Rickets in a Five Year Old Child

A—Cartilage shaft junction showing no evidence of defective lime salt deposition because proliferative activity is slowing *B*—Trabeculae of shaft showing in contrast osteoid borders

THE RELATIONSHIP OF MARBLE BONE DISEASE AND RICKETS

Now a word about marble bone disease (osteopetrosis) and about a feature of it that I think has not been suggested before It has been recognized that marble bone disease is associated with rickets in most cases (Figure 58) Dr Kramer has studied the calcium and phosphorus metabolism in at least two cases of marble bone disease[193] We interpret marble bone disease as resulting from virtually the complete cessation of destruction of bone In other words the cartilage matrix impregnated with inorganic materials *plus* the organic matrix impregnated with inorganic materials are not removed It is important to realize particularly in any

[19] McLean F C and Bloom W Calcification and Ossification Calcification in Normal Growing Bone *Anat Rec* 78 333 (1940)

[193] Pincus J B Gittleman I F and Kramer B Juvenile Osteopetrosis Metabolic Studies in 2 Cases and Further Observations on Composition of Bones in this Disease *Am J Dis Child* 73 458 (1947)

Fig 57 Osteoid in the Shaft of Bone of a Rapidly Growing Two Month Old Premature Infant

A—Cartilage shaft junction showing no evidence of defective deposition of inorganic materials *B*—Cortex of shaft showing in contrast a narrow but definite border of osteoid

studies of calcium and phosphorus metabolism in children that the body has a tremendous store of inorganic materials which is continuously being made available by normal destruction to be deposited in new matrix whether cartilage or bone In marble bone disease this inorganic material is locked up in the skeleton In other words it is not broken down and therefore not released to be deposited in new bone matrix and new cartilage matrix which is being formed and which is ready to calcify If the amount of inorganic material which can be absorbed is not sufficient in the face of this lack of the normal turnover which should be present we see a marked rachitic change such as in the rib in Figure 58

Shorr Is there any evidence of failure to develop osteoclastic activity

Follis There are osteoclasts present but not in excess

Shorr Are there any circumstances in which an effort has been made to induce them by demineralization or any procedure of the sort that would call them out?

Urist Yes has an effort of this sort been made as a treatment of the disease?

TABLE XXVI

The Pathogenesis of Rickets and Osteomalacia

I *Disturbance in Balance of Matrix Production and/or Destruction and Deposition of Inorganic Elements*
- 1) Normal rapid matrix formation especially in the premature
- 2) Healing fractures
- 3) Healing scurvy
- 4) Healing osteitis fibrosa
- 5) Marble bone disease

II *Disturbance in Absorption of Calcium and/or Phosphorus*
- 1) Calcium
  - a) Dietary lack
  - b) Change in pH of intestinal contents
  - c) Formation of insoluble complexes (PO_4 citrate oxalate phytin)
  - d) Protein content of diet
  - e) Diarrhea
  - f) Steatorrhea
  - g) Vitamin D lack
    - aa) Dietary
    - bb) Diarrhea
    - cc) Steatorrhea
    - dd) Absence of bile and pancreatic juice
    - ee) Phytin content of diet
    - ff) Impaired formation in skin
    - gg) *Increased threshold for effect*
- 2) Phosphorus
  - a) Dietary lack
  - b) Change in pH of intestinal contents
  - c) Diarrhea
  - d) Formation of insoluble complexes (Ca Sr Be Pb Fe Al)

III *Excess Excretion of Calcium and/or Phosphorus*
- 1) Renal disease
  - a) Glomerulo tubular (pathogenesis of bone disease not clear)
  - b) Tubular
    - aa) Increased phosphate excretion (phosphate diabetes) with or without glycosuria
    - bb) Fanconi syndrome (phosphaturia glycosuria amino-aciduria)
    - cc) Renal acidosis (phosphaturia kaluria defective bicarbonate absorbtion [?])
  - c) Acidosis
  - d) Idiopathic hypercalciuria
- 2) Lactation and pregnancy

IV *Obscure*
- 1) Vitamin D intoxication
- 2) Tumor cells in marrow spaces

Follis I do not think so. Do you know, Dr. Park? Has there ever been any effort made clinically to stimulate osteoclastic resorption? Of course the bones are in a demineralized state as it is. The condition apparently is not leading to any excessive destruction. Osteopetrosis is really the only disease, the only generalized disease in the human with which we are familiar in which this situation seems to hold. However, one can and does produce this same picture in certain species, for instance in the rat, as the result of either estrogen therapy[1] or cortisone therapy[2]. The rat is a peculiar animal in those two instances and no other species that I know of responds in this way.

Shorr How related is it to the situation in the bird?

Follis In the bird?

Shorr Yes, during the period when birds increase the density of their bones?

Follis Well, you see, this is bone which is already formed. There is no new formation, at least as we interpret it, in marble bone disease. Everything that is formed is normal, but none of it is destroyed.

Albright Is this unique or is it characteristic to have osteomalacia with osteopetrosis?

Follis Patients with osteopetrosis have varying degrees of rickets and I think this probably is related to the varying degree of involvement. Some individuals apparently are able to destroy bone in part.

Reifenstein What is the effect of increased calcium intake on the osteomalacia component of osteopetrosis?

Kramer We showed healing of the rachitic process.

Follis I should think if you could increase the intake so that the individual did not have to worry about the lack of breakdown, then he could go into positive balance.

Reifenstein But you do see cases of osteopetrosis without the osteomalacia component?

Follis I have never seen osteopetrosis histologically without osteomalacia or rickets in children.

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[1] Day, H. G., and Follis, R. H., Jr.: Skeletal Changes in Rats Receiving Estradiol Benzoate as Indicated by Histological Studies and Determinations of Bone Ash, Serum Calcium and Phosphatase. *Endocrinol.* 28: [illegible] (1941).

[2] Follis, R. H., Jr.: Effect of Cortisone on Growing Bones of the Rat. *Proc. Soc. Exper. Biol. and Med.* 76: 72[illegible] (1951).

Fig 58 Marble Bone Disease (Osteopetrosis) and Rickets

A—Costochondral junction showing marked deformity as a result of rickets and greatly increased density in the shaft *B*—Higher power magnification of *A* showing increased density of the shaft and three materials osteoid borders *(o)* bone (note osteocytes) and cartilage matrix *(c)*

Bartter Is this a purely histological observation or do these patient have the serum abnormalities that go with osteomalacia and rickets?

Kramer Oh yes They have a moderately low calcium and a very low phosphorus

Robinson What about the phosphatase

Kramer I do not remember off hand

Urist The serum alkaline phosphatase level is normal

Follis I do not know

Gutman In the adult form of osteopetrosis the serum calcium and inorganic phosphate levels are usually within normal limits and the serum alkaline phosphatase level usually is also high normal or only moderately elevated In some instances there may be a slight but apparently significant increase in serum acid phosphatase activity as yet unexplained

Kramer Is that the generalized picture?

Gutman Yes with multiple bone involvement

Engel How do you determine that the bone of marble bone disease is normal bone?

Follis It just looks normal We might as well stop now and as we indicated before make it plain again that although we are looking down the microscope and think we are in a position to make the final decision the changes that we see are really very gross ones Obviously there are changes which are going on long before one begins to see osteoid like that in Figure 58 and before one begins to see evidence of excessive destruction I am certain

Shorr My question is this can the turn over of lime salts and osteoid tissue going on normally in bone be attributed entirely to osteoblastic osteoclastic activity or to processes which do not involve the action of these cells?

Follis My own personal feeling is that osteoclasts are not the only mode of bone destruction osteoblasts destroy bone just as well There is a balance between constructive and destructive activity probably by the same cell What regulates that balance I do not know But you see a somewhat analogous situation in osteogenesis imperfecta where the osteoblast does not make any matrix In that disease there is plenty of cartilage matrix impregnated with inorganic material but there is no bone matrix on that cartilage matrix framework

Sullivan, T J Gutman E B and Gutman A B Theory and Application of the Serum Acid Phosphatase Determination in Metastasizing Prostatic Carcinoma Early Effects of Castration *J Urol* 48 426 (1942)

Park I think it is worth pointing out that in marble bone disease frac tures occur very commonly and when these trabeculae are fractured yo see a great deal of evidence of bone destruction

Follis There is another sixty four dollar question A similar situatio is found in osteogenesis imperfecta as soon a fracture occurs healin is seen The osteoblasts normally cannot make matrix but as soon as ther is fracture they are perfectly able to make an excessive amount so muc so that sometimes one may think there is a bone tumor present

Kramer There is evidence that the bone in marble bone disease is dif ferent from the bone of normal children of the same age

Follis However there is one point which I think you have to realiz when you say that Your chemical analyses of marble bone are includin considerable amount of cartilage matrix impregnated with inorganic ma terials

Kramer That is true but we took pieces of bone from different part of the long bone and also from different parts of the flat bones and I hav the analyses—

Follis And the analyses are different from ordinary rachitic bone?

Kramer No they are very much like ordinary rachitic bone

Follis Of course since the patients have rickets

Follis I do not know That is hard to say

To answer Dr Albright s question too Dr Copp showed us two years ago the depo ition of radioactive calcium in rachitic bone and demonstrated that it went very rapidly right through the osteoid borders so I do not see why it cannot come out also

Neuman As a matter of fact the radiocalcium did come out It went in but it did not stay in

Follis That is right so that would answer Dr Albright s question I think we pointed that out at the time as a matter of fact

DISTURBANCES IN ABSORPTION AND EXCRETION OF MINERALS

Now as Dr Howard indicated in his presentation at this conference we have to consider those conditions in which there are disturbances in the ab orption of calcium and/or phosphorus and also those situations in which there is an excessive excretion of calcium and phosphorus (Table XXVI) We have placed in this table al o two obscure conditions (vitamin D intoxication and tumor cells in marrow spaces) and I hope we can get a little discussion about them at the end of the session I do not think it is nece ary to go into all of these conditions with disturbances in the ab sorption of calcium which I think were referred to in large part pre viously I do want to say something about rickets as one sees it clinically and at autopsy and to say a word about vitamin D We shall not take up in detail any of these situations which have to do with phosphorus save one I thought it might be interesting to show you an example of what is probably conditioned phosphorus deficiency in association with lead ingestion in children

For the last few year Dr Park and I have been carrying out a study of the incidence of rickets at autopsy Figure 59 summarizes our results to date on a large series of autopsies at varying age periods up to 14 years Rickets begins very early at least there is microscopic evi dence of it we have seen lesions as early as the second week The incidence rises hit a peak about the eighth month and then begins to fall But one finds microscopic evidence of rickets in the sense of osteoid borders such as in the section I showed of the five year old child (Figure 56) even as late as the fourteenth year of life These children of course have var ious diseases which in some instances do seem to affect the development of rickets

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Follis R H Jr Jackson D Eliot M M and Park E A Pre alence of Rickets in Children Between Two and Fou teen Years of Age *Am J Dis Child* 66 1 (1943)

Armstrong Do you have any idea how long would be required for rickets to develop beginning with the status of the skeleton which does not exhibit these histological abnormalities?

Follis As I have said we have seen it as early in infancy as the thirteenth or fourteenth day

Park You might just point out that you have never seen it in newborn babies

Follis We have never seen it in newborns or in stillborns in this country Of course it has been described at birth in China

Armstrong So a short period of illness might contribute very significantly to rickets

THE RELATIONSHIP OF GROWTH AND DIETARY RICKETS

Stearns Do you have the history of the growth and feeding of these babies? I asked that Dr Follis because Dr Park will remember back in the thirties that when Dr Elliot looked over our x rays of the normal infants that we had been studying she declared that all of those with rapid growth had rickets We had the data on the blood values and on their retention values and if rickets is a disturbance of mineral metabolism they could not have had rickets but merely were growing very rapidly I think those x rays and the data went all over the country and the final consensus seemed to be that for Dr Elliot s Grade B rickets you had to know the history and that otherwise the irregularities might be due merely to rapid growth if the child was very well fed and growing very rapidly

Follis I think that is an important point and one we have already indicated In order to have bone disease at least many of these diseases one has to have growth For instance one does not see changes at the cartilage shaft junction in the older child because the child has stopped growing one does see evidence morphologically in the shaft which one would not see in the x ray Rickets has to be present—and I hope you will say something about this Dr Park—in fairly severe degree *histologically before* it manifests itself radiologically

Stearns But apparently some changes which simulate those of rickets do manifest themselves radiologically in the healthy very rapidly growing baby who has high mineral stores

Park I think the x ray method of diagnosing rickets is extraordinarily fallacious both ways don t you agree Dr Follis?

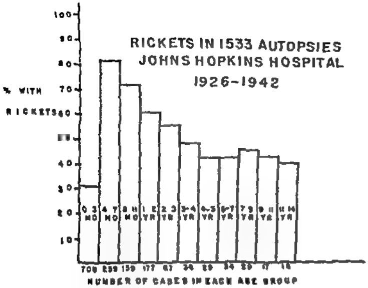

Fig 59 The Incidence of Rickets at Autopsy in the Johns Hopkins Hospital Series

The e fin lings are d cu sed in d tail el ewhere

Follis I would rather have you as a clinician say that

Park You cannot recognize rickets as Dr Follis has said unless it has undergone a very considerable degree of development all sorts of variations in the outlines of the ends of the bones are seen in the x ray which simulate the changes that take place in rickets I think that Dr Elliot s criteria probably do not hold Dr Stearns

Follis Of course we see the opposite as I think you indicated Dr Stearns If children who might be expected to show rickets from their dietary history do not grow they do not show rickets In a group of individuals who spent most of their lives in the hospital and in whom we knew the vitamin D intake or knew that the vitamin definitely had not been administered we have seen case in which although there had been

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Foll s P H Jr Park E A and Jackson D The Relati n h p of Vitam n D Adm n t at n to the Prevalence of Rickets Obs r e l at Aut p y During the First Two Years of Life B ll John Hopk ns Ho p to be publ h d

no vitamin D given rickets was not present as one might have expected it to be Many of such individuals did have evidence of growth arrest that is we could tell under the microscope that the proliferative activity of the cartilage had decreased

THE RELATIONSHIP OF VITAMIN D INTAKE AND DIETARY RICKETS

Dr Park and I have been particularly interested in studying a relatively small group (although it is now over a hundred) of children whose vitamin D intake was known because it had been administered in the hospital In our series of children who received no vitamin D and then came to the hospital and received vitamin D for varying periods we can see the development of Muller s sign in the costochondral junctions and get some idea as to the *effectiveness of the treatment*

Figure 60A is from a six month old child who received 1000 units of vitamin D daily for nine days The point which has impressed us is the variability of the reaction Such an impression fits in very nicely with the variations in calcium absorption which Dr McCance mentioned earlier at this Conference and probably with the variations in the vitamin D responsiveness of the individual There are all gradations in the way in which a given individual reacts to vitamin D and the possibility exists as I pointed out to Dr McCance in a private discussion that there may be some individuals who probably will behave much like rats (and he agreed that there were many individuals who behaved like rats!) as far as their metabolism of calcium and phosphorus was concerned In other words there may be people who need very little vitamin D and therefore are like rats and there may be others who need much more vitamin D and fall into the vitamin D resistant rickets group

Figure 60B shows another example of the healing effect that is apparent after 25 days in a child 18 months old who received 2200 units of vitamin D daily for that period of time It is interesting that the healing is just beginning to manifest itself in the x rays and not in the clinical x rays but in the postmortem x rays taken of the bone after its removal from the body We can pick up these healing effects a great many days earlier histologically than we can detect them clinically It takes about three weeks does it not Dr Park for the changes to manifest themselves in the clinical x rays?

Park That is right

Follis I am certain that we can pick them up in much less time histologically

Fig 60 The Effect of Vitamin D Therapy on Dietary Rickets in Children

A—Bone section from a six month old infant treated for 9 days with 1000 units of vitamin D per day *B*—Bone section from a 18 month old child treated for 25 days with 2[illegible]00 units of vitamin D per day

Park I believe Dr Follis that you have to take into consideration the dosage of vitamin D With enormous dosages I think we have seen healing in seven days With about 1200 units of vitamin D as cod liver oil we quite regularly observed it about the twenty first day

Follis There are certain situations as you obviously must have thought about during previous discussions of this Conference which may lead to defects in vitamin D absorption one of these of course is congenital obstruction of the bile ducts Figure 61 is from a five month old i

who received daily 2200 units of vitamin D for all of its life You can see that that was not enough as one might have expected to protect it against the development of rickets

Of course one finds exactly the same situation in fibrocystic disease of the pancreas . Such individuals are not absorbing vitamin D and they probably are losing more calcium in the stools because of the excessive fat which is present

THE RELATIONSHIP OF LEAD POISONING AND RICKETS

We have had 60 odd cases of lead poisoning in children at autopsy I do not know why only Baltimore seems to have lead poisoning It is fairly prevalent still because virtually not a Summer goes by without our having at least one case of lead poisoning at autopsy Figure 62A is from a 24 month old child showing marked rachitic changes much more than is usual for this age There is also an excessive amount of matrix impregnated with inorganic materials which Dr Park described [64] as you all know a number of years ago as a peculiarity of lead poisoning In many cases there is rickets as well Our theory is that lead in the stool combines with phosphorus to form an insoluble phosphate This may lead to a low phosphorus type of rickets Unfortunately we do not have any metabolic studies Perhaps Dr Kramer has

Armstrong Is it possible that an amount of lead could be ingested which would bind significant amounts of phosphorus? This is incomprehensible to me

Follis These children have a very large quantity of lead in their intestines

Armstrong Yes but thinking of the amount of aluminum hydroxide that has to be given to do this—

Follis I just threw that idea in as a possibility I do not know how to explain the rickets The children with lead poisoning have a much higher incidence of rickets than the population as a whole It is one of the few diseases in which there seems to be some relation to the presence of rickets in the older child There is another peculiar situation and maybe Dr Park will say something about it virtually all of the children with lead poisoning who come to autopsy die in the Summer Their lead poisoning at autopsy at least has a distinct seasonal variation

Armstrong This might be explained by the source of their lead

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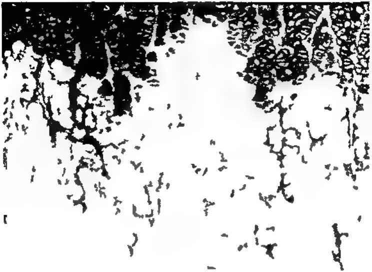
Park E A Jackson D Goodwin T C and Kadji L X Ray Shadows in Growing Bones Produced by Lead Their Characteristics Cause Anatomical Counterpart in the Bone and Differentiation *J Pediat* 3 265 (1933)



F g 61 I cket n a F e Month Old Ch ld D g of Conge tal I l ary Atre a

The o ho d al u t on h ws v d loped ke s n sp e of he da y a m n a on of 2 00 u s of v am n D e b

Fo l s No I tl k the more l kely explanat on s the sunl gl t

P ter Ho v does tl e sunl gl t expla n t

Foll s It m gl t ncrease tl e ab orpt o of lead The cl ldren d e of en ephal t

Sol I W th tan n D take tl ere a n arked r se of blood lead at least n exper n e tal an mal and tox cat on fron lead supposed to be propo t onal to the con entrat on of c rculat ng lead or at least that a factor Moreo er man ears ago when we stud ed lead r ckets (r ckets p od ced by putt ng a large amo nt of lead to tl e d et) t seen ed to

S be A E C on O and K ame B Influ e of V am n D n Exp m a Lead Po on ng *P o So E p B ol a d Med* 38 433 (1938

Sol l A W x] r I B Pe o ky D anl K ame B Influen e of D e a y Ca um and Ph ph us upon Act n f V am n D Expe nental Lead Po n ng *J So Exp B a d M d* 38 435 (1938

S bel A E Yu ka H Pe e D D a d K m B The B ochem l Be ha of Lead I I flu n e of Ca um, Pho p o u and V am n D o Lead Blood and Bo e *J B ol C* 13 3 1940

S be A E Yu ka H and K me P Influen e f Cal um P o pho u and V am D n Lead n Blo d a d I o e D ng D l ad g *J B C e n* 140 CXX (1941)

Fig 62 Rickets in a Two Year Old Child Dying of Lead Encephalitis

A—Co tochondral junction showing rickets which is acute and very severe for this age *B*— Lead line zone which is composed of impregnated cartilage matrix and bone both in excess Note large o teocla ts

that the calcifiability of such bones *in vitro* was much less than that of control bones with rickets and the chances are that lead affects the calcifiability of the matrix directly besides having some effect on the intestinal tract

Follis Possibly The only point against this is that you see rickets in only about half of the cases

Sobel But you have very small concentrations of lead in most of your cases

Follis I do not have the slightest idea of how much lead these children ingest Maybe you have an estimate Dr Park of how much lead is ingested?

Park I do not know But it is a very common occurrence with a baby who has been accustomed to eat paint to take an x ray picture of the abdomen and find it just filled with the shadows which are cast by the paint This is one method used sometimes to recognize paint ingestion

Follis I do not know whether you see much lead poisoning in England Dr McCance I recall a couple of reports I have noted in the English literature

McCance What about the kidney function? What is the level of serum phosphorus in these children? By any chance do they have renal rickets?

Harrison No the level of serum phosphorus is not strikingly reduced nor are there any changes in the level of serum calcium The patients do have consistent disturbances in renal function manifested by albuminuria occasionally by some microscopic red cells by casts in the urine and sometimes by glycosuria But if phosphaturia is a problem it does not manifest itself by a reduction of the level of serum phosphorus

McCance I should expect the serum phosphorus to be raised That is what happens in renal rickets

Follis In these individuals who have lead poisoning—and this is negative evidence of course—there is nothing in the kidneys histologically with the exception of inclusion bodies [39]

McCance I am astonished that you should be able to see lead radiologically in the abdomen

Follis Fluoroscopy is a favorite way to show it in the Harriet Lane Home of the Johns Hopkins Hospital is it not?

Park I am not certain that the shadows are cast entirely by the lead Dr Kramer are there other products in paint which would be radio opaque?

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[39] Blackman S S Jr Intranuclear Inclusion Bodies in the Kidney and Liver Caused by Lead Poisoning *Bull Johns Hopkins Hosp* 58 384 (1936)

Harrison. There are other substances present besides lead and very often the children ingest plaster along with their lead and of course the calcium sulfate casts a very strong shadow

Bartter. How many milliequivalents of lead might you find in the whole gastrointestinal tract of one of these patients Dr Follis?

Follis I cannot answer that Can you Dr Harrison? How many milliequivalents of lead might you find in the gastrointestinal tract?

Harrison I will tell you the answer to that in six months There is no answer at the moment

Butler In a child who has recently ingested paint the x ray shows little flakes of opaque material scattered throughout the bowel I cannot imagine that these would amount to a great deal of lead and I doubt whether the amount would interfere with the absorption of phosphorus from the intestine

Shorr Are these children excreting phosphorus in the urine?

Butler They do not have any renal disease that results in any elevation of the nonprotein nitrogen or the phosphorus levels in the serum

Harrison And the serum phosphorus levels are not reduced

Follis The trouble is of course that these children are admitted with convulsions and are usually dead in 48 hours (if they are going to die) therefore it is somewhat difficult—isn't that true Dr Harrison?—to do metabolic studies on them

Harrison Yes that is right

Butler Yes if they have acute encephalitis But on the other hand, you have patients with mild or chronic cases of lead poisoning

Follis Then they do not come to my department of pathology!

Shorr Dr Harrison I would just like to add with respect to the possibility that it is the ingestion of the lead that diverts the phosphorus that unless you have a patient who is receiving so much phosphate binder that there is no excretion of phosphorus from the urine you can expect at least on the basis of aluminum gel experiments that they will remain in positive phosphorus balance You can reduce the urinary phosphorus excretion from 1000 mg to 200 or 100 mg per 24 hours and the patient will still be in positive phosphorus balance I would therefore question very much the diversion you are achieving through the gastrointestinal tract

Follis How about the excretion? Have you studied that?

Harrison In the stool?

Follis No in the urine as was suggested

Harrison No we have not

Rubin On this same point it might be well to bear in mind that in experimental lead poisoning the transport of lead is from the intestinal tract to the soft tissue primarily the liver within a matter of hours and from there to the marrow in the bone over the period of the next few days so that one would be suspicious in thi situation of a direct effect of lead at the location that you are considering

Follis But this problem concerns children who have been eating lead for several months

Rubin Exactly This is just the situation in which you would expect to find most of the lead in the bones

Follis Yes of course The seasonal incidence is very interesting and as I suggested may be related as Dr Sobel said to the excessive absorption of lead which may take place

Sobel No I said something else Vitamin D raises the blood level on a given intake not as a result of absorption

Follis Not due to absorption?

Sobel This can happen even when you do not have lead in the diet when there is lead in the bones

You may have read the book by Ellsworth in which he describes a diary account of the activities of some shipwrecked sailors in the Arctic Whoever wrote that diary was a very good clinician The men were eating from tin cans (which in those days were sealed with lead) and they were getting along well until along came the six month long day The sailors went out and sunned themselves and for a time felt very good But the diarist writes Our joy did not last long because we soon came down with the palsies and what not which the ship s doctor diagnosed as lead poisoning The source was not far to seek because the sailors boiled the tin cans which were lead sealed in the pot But the question is why did the lead poisoning become apparent during or after the sunshine It is my interpretation that since the men were irradiated at that time rather intensively the endogenous vitamin D formed by the sunshine raised the blood lead level we know that the circulating blood lead concentration and the toxicity seem to be related [2 3 203]

Follis You mean there was no additional ingestion?

Sobel At that point the ingestion did not suddenly change

Follis Have you experimental evidence that when there is no ingestion of lead vitamin D raises the blood lead concentration?

Sobel Yes We have not published that portion of our studies but we did the others Vitamin D raises the level quite considerably as a matter of fact

Rubin The clinical symptoms are associated with the shift of the lead from the bone to the soft tissues This soft tissue and neural fixation of lead appears to precede the development of the encephalopathy

Follis That is not what kills these children The brain lesions kill them

Sobel As far as the incidence of lead poisoning is concerned I think you would see more everywhere if you took the trouble to look for it I remember when we were intensively doing lead investigations there were many cases When you do not look for them there appear to be no cases or they are difficult to find

Follis It does make us wonder why we see so much in Baltimore and nobody elsewhere seems to see nearly as much at least at autopsy

Harrison These children come from homes that have not been painted for about twenty years

Shorr But they play about the neighborhood

Harrison The parents may paint the cribs

Rubin It is interesting that twenty years ago people were using lead paints on the interior of houses in Baltimore

Butler The high incidence may be because your houses have not been painted for twenty years and hence the paint that the children are chewing in those houses is old paint

Follis You can still buy new paint in Baltimore

Butler Yes but the very good white lead paint that was used in those days is no longer obtainable

Follis A state law was passed but the legislators had to rescind it

Sobel Dr Butler when my children were young I tried all over New York to find paint that was lead free While theoretically there should

have been such paints I could not get any Later when we were painting fences we found some white casein paint but it was not satisfactory Finally we were forced to use lead paint

Follis It is interesting as Dr Park has pointed out to me that the incidence of lead poisoning increases at the time the incisors appear

Urist Dr Follis exactly what is the lesion at the costochondral junction in lead poisoning?

Follis In the uncomplicated cases in the cases in which there is no rickets there is an extremely dense deposition of matrix impregnated with inorganic material covered by bone (Figure 62B)

Butler What is the serum alkaline phosphatase level in lead poisoning?

Follis Dr Harrison do you know?

Harrison I cannot tell you offhand My recollection is that the level is not elevated

Butler That is my recollection The toxic effect might stop the cellular activity

Follis It is not a toxic effect because these patients are able to make bone matrix The osteoid indicates that that matrix apparently has been deposited or appeared there during the period when they have been ingesting the lead

Butler Then wouldn t you expect to have the serum alkaline phosphatase level elevated?

Follis I would imagine so

Butler I would too and yet I do not recall it

Harrison I do not remember the figures

Follis Unless there is some effect of lead ion on the phosphatase in action in the blood such as beryllium exhibits *in vitro* Do you know anything about that Dr Gutman?

Gutman No

Henneman Poisoning of the alkaline phosphatase activity might produce the histological appearance of rickets

Follis *In vivo*? How do you poison alkaline phosphatase?

Henneman There is a recently described[2] syndrome of hypophos

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[2] Rathbun J C Hypophosphatasia A New Developmental Anomaly *Am J Dis Child* 75 822 (1948)

phatasia in which the serum calcium and phosphorus levels are normal the bones are osteomalacic and the alkaline phosphatase content of the blood the bones and the intestine is markedly reduced

Follis Yes I am familiar with this report That situation is of unknown etiology I know of no way and if anyone does I would be interested in hearing of it in which you can inactivate alkaline phosphatase *in vivo* Do you Dr Gutman?

Gutman No except that in radium poisoning the serum alkaline phosphatase level may be low apparently as a result of inactivation of osteoblasts

Park There is reason to think that there is an hereditary factor in the cases of hypophosphatasia

Bartter Dr Follis have you not suggested that lead may suppress the alkaline phosphatase activity?

Follis I did not make that suggestion no. Please do not put that one down for me

Henneman But if it is true that these children with lead ingestion have normal serum calcium and phosphorus levels and do not have an elevated alkaline phosphatase level then the possibility exists that lead may produce osteomalacia via poisoning of the alkaline phosphatase

Follis True if there are enough studies on such cases as these with rickets in which the phosphatase is normal

Sobel I think that in lead rickets you are dealing with the inactivation of the local matrix just as with strontium or beryllium rickets[11] and

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[11] Sobel A E Goldfarb A R and Kramer B Studies of Incurable Rickets I Respective Role of the Local Factor and of Vitamin D in Healing *Proc Soc Exper Biol and Med* 31 869 (1934)

[12] Sobel A E Goldfarb A R and Kramer B Studies of Incurable Rickets II Role of the Local Factor and of Viosterol in the Pathogenesis of Rickets Due to Beryllium *J Biol Chem* 108 395 (1935)

[13] Sobel A E Cohen J and Kramer B The Nature of the Injury to the Calcifying Mechanism in Rickets Due to Strontium *Biochem J* 29 2640 (1935)

[14] Sobel A E Cohen J and Kramer B Phosphatase Activity and Calcification in Strontium Rickets *Biochem J* 29 2646 (1935)

[15] Sobel A E Goldenberg H and Hanok A Calcification IV Influence of Strontium and Magnesium Ions on Calcification *In Vitro* *Proc Soc Exper Biol and Med* 78 716 (1951)

[16] Sobel A E The Local Factor in Calcification *TRANS MACY CONFERENCE ON METABOLIC INTERRELATIONS* 2 113 143 (1950)

[17] Sobel A E Studies on the Local Factor of Calcification *TRANS MACY CONFERENCE ON METABOLIC INTERRELATIONS* 4 113 179 (1952)

that the condition is not necessarily due to the removal of the intestinal phosphate. The calcifying mechanism is injured locally so that at the usual humoral conditions of calcification one does not get the same degree of mineralization.

Follis: It seems to me a little strange that in cases of lead poisoning we do not see far more rickets than we do.

Sobel: This is because the degree of inactivation of the Local Factor I studied that year ago without pulling the findings— not as great in lead poisoning as in strontium or beryllium rickets but definitely does occur. That is, if we attempted to calcify in vitro the rachitic bones of animal fed lead they would not calcify as readily as the normal rachitic bone indicating that the calcifying power of the lead affected rachitic bones is not as good as that of the usual type of rachitic bones.

Engel: The lead could compete with calcium for the ground substance and combine with it instead.

Follis: True, but still there is lead in this area. It has been shown to be present chemically.

Sobel: Just the fact that there is lead in the area does not alter the situation. In fact it merely proves the possibility.

Follis: Perhaps the lead can substitute for calcium. Dr. Neuman has told me that uranium can substitute for two calcium atoms. It may be that lead also can substitute for two calcium atoms.

Neuman: I would guess it must be because it is so large.

Sobel: That is in the final crystal lattice before —

Follis: No, this is on the surface of the crystal.

Sobel: In the initial process of calcification in the cartilage matrix, if lead combines with the same spot that calcium will go, it can interfere to some extent, at least comparatively with the degree of calcification.

Follis: Yes, but still that might not necessarily give you the histological picture of rickets. The bone would be mineralized but it would be plumbified instead of calcified.

Sobel: Yes and no. In the case of strontium rickets which McCollum originally reported[8] strontium combines with the matrix but also it pre

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[8]Shipley, P. G., Park, E. A., McCollum, E. V., Simmonds, N., and Kinney, E. M.: Studies on Experimental Rickets. XX. Effects of Strontium Administration on the Histological Structure of the Growing Bones. *Bull. Johns Hopkins Hosp.* [illegible] 216 (1922).

vents the deposition of calcium phosphate Thus McCollum obtained incurable rickets Strontium is an extreme case but with lead you might have a mild example of the same phenomenon

Handler Dr Sobel is suggesting that it is a catalytic spot onto which the lead falls a spot which is supposed to take care of thousands of calciums Once the lead gets on it does not come off and one lead therefore competes with thousands of calciums

Follis The histological picture is shown in Figure 62B Just beneath the cartilage there is a very very dense band (Dr Park's lead line) which you see in x rays This is composed of cartilage matrix which apparently is impregnated with calcium and phosphorus carbonate and probably lead surrounded by a dense zone of bone and excessive numbers of giant cells (osteoclasts) which seem to be more or less impotent as far as their being able to destroy this area is concerned and so it persists This to some extent resembles the picture I could show you (if there were no giant cells) which looks like marble bone disease in a given area Once you examine a few fields under the microscope you begin to see the giant cells and then you know that you are dealing with lead poisoning We have found cases at autopsy that were not suspected clinically

Urist Are these foreign body giant cells osteoclasts?

Follis I have no way of differentiating between the two They are not phagocytic although there is one in Figure 62B that seems to have placed its arms around a spicule which might indicate that it is phagocytic

INCREASED EXCRETION OF MINERALS WITH RENAL DISEASE

The last group of situations (Table XXVI) in which there is increased excretion is usually associated with renal disease I do not think I have to spend much time on these possibilities in which there is glomerular tubular disease of varying degrees First there is glomerular and/or tubular disease and then primarily tubular disease which may be related to excessive phosphate excretion and possibly to increased resistance to vitamin D In addition there is the Fanconi syndrome (with which I know you are all familiar) and renal acidosis The possibility that this latter condition may be related to defective bicarbonate absorption recently has been discussed [219] Then there are the cases in adults which Dr Albright and Dr Reifenstein [88] described of idiopathic hypercalciuria Lastly I

[219] Latner A L and Burnard F D Idiopathic Hyperchloremic Renal Acidosis of Infants (*Nephrocalcinosis Infantum*) Observation on Site and Nature of Lesion *Quart J Med* 19 285 (1950)

would like to say a word about vitamin D intoxication and the presence of tumor cells in marrow spaces

In chronic glomerular nephritis in children and in adults one runs into excessive amounts of osteoid and hence we believe that the terms "renal rickets" or "renal osteomalacia" are justifiable ones. The cause for this osteoid is not clear. The patients have deranged serum concentrations of calcium and phosphorus as you know with a decrease in calcium and an increase in phosphorus but it is very very difficult to know which serum has calcifiable properties and which has not. Through the kindness of Dr Howard and his associates we have been able to study by *in vitro* calcification the properties of certain nephritic sera. You do not know at a given concentration of calcium and/or phosphorus whether you are going to get calcification of the rachitic tibia or not. Figure 63A is from a child three years old dying of chronic renal insufficiency with virtually pure rickets. There are all gradations from this picture all the way to that of children or adults such as Dr Albright has described who have primary excessive destruction or osteitis fibrosa. What are the factors that regulate the type of bone involvement? We think the duration perhaps and the degree of renal insufficiency are important although it is extremely difficult to be certain because chemical studies are not available.

Butler: How about age?

Follis: You mean as to whether it occurs more frequently in the younger children?

Butler: Yes

Follis: I think that is an important factor as we have pointed out[1] you do see osteoid more frequently in the younger growing child. There again the renal insufficiency may not have been present very long. But one does see osteoid in the adult as well[2] so that osteomalacia is certainly present in association with renal disease. Figure 63B is from a child I think four or five years old who has had edema and evidence of chronic nephritis for a year and a half. In addition to the osteoid you begin to see evidences of osteitis fibrosa.

Henneman: Is there osteomalacia there too?

Follis: As I said in addition to osteitis fibrosa there is rickets as well or if you prefer the term osteomalacia as well.

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[1] Follis, R. H., Jr.: Renal Rickets and Osteitis Fibrosa in Children and Adolescents. *Bull Johns Hopkins Hosp* 87 593 (1950)

[2] Follis, R. H., Jr. and Jackson, D. A.: Renal Osteomalacia and Osteitis Fibrosa in Adults. *Bull Johns Hopkins Hosp* 72 73 (1943)

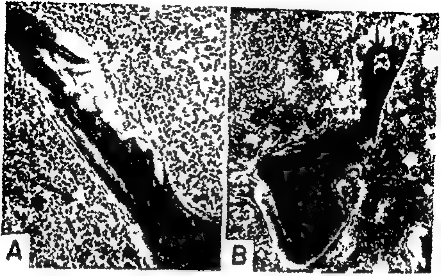

Fig 63 Rickets in Children with Chronic Nephritis

A—Trabecula showing osteoid from a five year old girl dying with chronic pyelonephritis that was of one year's duration clinically *B*—Trabecula showing osteoid from a four year old girl dying of chronic glomerulonephritis that was of at least 1½ years duration *Note the beginning focus of osteitis fibrosa (arrow) in the trabecula*

Figure 64 shows that there may be excessive osteoid in the adult If bone lesions are present in adults I would say that the osteitis fibrosa comes at a later stage than does the osteomalacia

Gutman Does the osteitis fibrosa imply hyperparathyroidism?

Follis We use the term osteitis fibrosa to mean microscopic evidence of excessive destruction

Gutman But you do not imply that there is excessive secretion of parathyroid hormone?

Follis No osteitis fibrosa implies a considerable amount of bone destruction which may be associated with a number of reasons or causes

Gutman May I ask one rather silly question? Is rickets as seen by the pathologist an entity or is it a syndrome?

Follis It is extremely difficult to define rickets Some people I think would like to define rickets (although I do not particularly want to) as a defective mineralization or a defective deposition of inorganic materials

Fig. 64. Zone of Osteoid in the Vertebra of an Adult Dying with Chronic Nephritis

as a result of defective dietary intake of calcium or phosphorus or vitamin D. I think it is impossible to distinguish microscopically evidence as to what factors (such as we have been discussing here) may have led to the condition, so my definition of rickets is, as we have indicated, that it is just a state of defective deposition of inorganic materials in cartilage and/or bone matrix from any one of the possibilities that we have discussed (Table XXVI). At least you cannot tell these conditions apart under the microscope. Would you, Dr. Park, care to comment on that point for Dr. Gutman?

Park: I think that exactly what you have said sounds satisfactory to me.

Butler: I would agree with your definition but I would like to add one point. You talked about renal rickets as arising from the derangement of the serum calcium and inorganic phosphorus levels. I would like to suggest that the main factor in causing renal rickets in chronic nephritis is not perhaps the change in the serum calcium and inorganic phosphorus level but is the acidosis. I make that statement because of the fact that you find renal rickets in children who have no decrease of the serum calcium level and no marked decrease of the serum inorganic phosphorus level but who do have chronic acidosis. You can correct the acidosis and all of the rickets heal.

Follis I put in acidosis Dr Butler as a special heading in Table XXVI mainly for Dr Reifenstein but I see that I have added it for you too

THE RELATIONSHIP OF VITAMIN D INTOXICATION AND RICKETS

I think it was three years ago ⁽¹⁾ that we brought up the question of vitamin D intoxication and pointed out to this group as others had indicated in the literature that hypervitaminosis D is associated with rickets If you give enough vitamin D you do not produce changes in the cartilage but you can produce an excessive amount of osteoid in the metaphysis (Figure 65) This osteoid appears in the face of high concentrations of calcium and phosphorus in the serum It seemed somewhat peculiar when we began to study it and it still does this morning as far as I am concerned Dr Harrison has done some citrate determinations on some of our experimental animals and finds I think that there is no rise —

Harrison No there is an elevation There is always a hypercitraticemia with vitamin D intoxication in humans as well as in rats

Fig 65 Metaphysis from a Rat Treated with a Large Amount of Vitamin D

The section is undecalcified and stained with silver nitrate to bring out the wide zone of osteoid

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Follis R H, Jr The Influence of Essential Nutrients and Hormones on Cartilage and Bone *TRANS MACY CONFERENCE ON METABOLIC INTERRELATIONS* 2 221 257 (1950)

Follis But that would not lead you to suspect that the calcium was being bound in any way

Harrison No The rise in serum citrate concentration is not sufficient to account for the findings

Follis In other words the possibility arose that in the presence of these high concentrations of calcium and phosphorus the calcium phosphorus might be bound in the serum in some soluble form and could not deposit in the matrix If so it is peculiar that calcium phosphate does precipitate in the kidneys and in certain areas—the heart and other tissues—but does not in the matrix apparently The question may also be raised is there anything unusual about the osteoid in vitamin D intoxication? I think Dr McLean made that suggestion several years ago We have found that you can calcify this osteoid if you use normal serum and leave the slices of osteoid in it long enough

Armstrong Is it possible that Dr Engel s mucopolysaccharide is present in the blood?

Follis Present in the blood? I do not know

Armstrong Or in the kidney?

Engel I think that one would assume at least from a speculative standpoint that the matrix here is altered that its calcium binding capacity is reduced and that the calcium ion spill into the serum Yet you can show by using the nomogram that it is possible to lower the calcium ion concentration of bone matrix while the calcium ion concentration of other tissue is elevated This would be conducive to calcification such as occurs in the heart

Follis Personally I think osteoid formation is due to a direct stimulation by vitamin D because you can get it on a low calcium and low phosphorus diet

Robinson Do you think that the rows of osteoblasts in Figure 65 mean that there is new osteoid?

Follis I think it is undoubtedly new osteoid because it actually forms only in the metaphysis where growth is taking place You do not get nearly as much down in the shaft

Urist Are these the bones of children? How old is the patient?

Follis Oh the patient is about a 0-gram rat [Laughter]

Stearns In France and Belgium apparently 15 mg vitamin D capsules are sold across the counter as good for whatever ails you They are bought

by the families of sick children and I have seen some of the x rays of such children Some of them have had as many daily as two or three of the capsules over a period of a few months This is really a very large problem One clinic has had 20 deaths from these capsules within a year All of the patients showed a *consistent* x ray pattern They had a very heavy dense band of calcification that reminded me of the bismuth lines we used to see in bones of children but the ossification of the cartilage centers was greatly delayed and the bone growth also was retarded

Follis The cartilaginous bone growth in these animals certainly was interfered with

Sobel Speculatively we may introduce another concept Dixon has demonstrated the presence of a citric acid system in osteoid [3] One of Nicolaysen s co workers this summer gave a paper [4]—Dr Neuman and Dr Kramer were there—in which he said that vitamin D directly stimulates citric acid production in bone If we combine these two facts it is possible the vitamin D causes an increased production of citrate locally in the bone and thus prevents the proper deposition of minerals The citrate would cause decalcification right in the bone and it would not manifest itself in the blood to the same degree in other words the local effects of the citrate could cause the picture rather than the systematic concentrations of the citrate throughout the body fluids

THE RELATIONSHIP OF LEUKEMIA AND RICKETS

Follis We are getting a little behind and Dr Armstrong probably is becoming worried so let us proceed

One sees also some peculiarities involving excessive amounts of bone matrix without inorganic material in certain diseases and osteoid formation in these conditions is a process the mechanism of which is unknown Figure 66 happens to be leukemia in a child Dr Park and I in a study of leukemia in children [5] have been impressed by the excessive amount of osteoid which may be seen We do not know how to explain it I do

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[3] Dixon T F and Perkins H R Citric Acid and Bone Metabolism *Biochem J* 52 260 (1952)

[4] Nicolaysen R The Interaction of Vitamin D and the Endogenous Factor in Calcium Absorption Remarks on the Mode of Action of Vitamin D Presented before the 2nd International Congress of Biochemistry Symposium on Fat Soluble Vitamins (July 23 1952)

[5] Follis R H Jr and Park E A Some Observations on the Morphologic Basis for the Roentgenographic Changes in Childhood Leukemia *Bull Hosp Joint Dis* 12/2 67 (1951)

Fig 66 Osteoid Borders along a Trabecula from a Child with Lymphatic Leukemia

The findings are discussed in detail elsewhere

not think there is any point in stopping longer than to call your attention to this phenomenon

Shorr When you say excessive osteoid do you mean the same thing as diminished calcium deposition?

Follis Yes Figure 66 is ricket' but I do not know how this condition was produced In some of these cases the individuals have received large or fairly large quantities of vitamin D and still showed this condition in the bone I think that the calcium and phosphorus metabolism should be studied in cases of leukemia in children Do you have any data on that Dr Butler?

Butler No

Follis Of course one sees excessive rarefaction but why the excessive osteoid material also?

Gutman Hypercalcemia occurs in some cases of acute leukemia in children and may be quite marked (12 to 15 mg per 100 cc in some of our cases) One sees evidence of bone rarefaction in roentgenograms of the bones in acute leukemia in children particularly the so called juxta-epiphyseal zone of rarefaction

Follis You see rarefaction and then zones of increased density which are areas as Dr Park and I pointed out a year or so ago where fractures have taken place [3]

Park In several cases at Harriet Lane Home Dr Follis serum calcium and phosphorus determinations were done and no abnormalities in the levels were found

Follis You would expect to find hypercalcemia in the presence of leukemia As I have indicated before in certain cases there is evidence of excessive destruction In leukemia particularly in children one finds three situation in bone 1) one may see osteoid 2) one may see excessive destruction or 3) one may find that the bone seems to have melted away You cannot discover any histological evidence for what is happening in the last situation except that the bone just is not there

Kramer There is periosteal penetration

Gutman I believe acute leukemia is one of the important causes of hypercalcemia in children However I have not encountered hypercalcemia in the few cases of leukemia in adults that I have tested

Bartter In Figure 66 is not there really very little rickets?

Follis It is a considerable amount for this age

Bartter Nine tenths of that bone is quite normal is it not?

Follis Yes The reason that I have presented Figure 66 is to stimulate interest in finding out how the bone lesion is produced how it comes about particularly if there is excessive destruction and inorganic materials are being liberated

Harrison Here again you may have the factor of abnormal metabolism of the cell in close contact with your bone matrix

Follis Yes We suggested once ten years ago when Dr Park and I were reporting this series [a] that the leukemic cells may be stealing the inorganic materials getting hold of them before they could reach the matrix

Harrison Or the leukemic cells may be changing the conditions locally so that the factors involved in the deposition of calcium in the matrix are altered or inhibited

Follis Yes so it is another instance of the complexities is it not Dr Neuman that we have to consider in these meetings?

Neuman I have been wondering because of the similar morphology of the fibroblasts the osteoblasts and the osteoclasts whether or not we have in these cells a structural form that is rather undifferentiated and has a good deal of adaptability in terms of its metabolic pathways and what not that if the oxygen tension is altered its metabolic pathways shift

so that in one case the cell will put out a considerable quantity of citric acid and in another case it will put out alkali or some such material—

Follis Or Versene

Neuman Yes Versene perhaps that is a good suggestion This phenomenon is seen in tissue culture work We tried for a long time to get tissue cultures of osteoblasts they would be very useful preparations to study But if we obtained a tissue culture that originally contained osteoblasts the osteoblasts either did not grow in which case they remain osteoblasts or if they did grow they became fibroblasts

Shorr Does anyone know how well oxygenated this crude bone marrow is? You may have a local acidosis

Follis I do not have the slightest idea

Robinson If you had a fracture with a piece of necrotic bone in it which disappeared without cellular activity you could make a critical observation

Follis I do not know as I will show you

Robinson I mean if you are considering chemical dissolution of bone without cellular activity

Follis It may be that the leukemic cells themselves have some mechanism for destroying bone

Henneman The rapid uptake of phosphate by the leukemic cells might cause a low phosphate ricket

Follis That is what we suggested[2] based on some work which was done by Lawrence and the California group twelve or fourteen years ago

Henneman If the leukemic cells took up an excessive amount of phosphorus resulting in local bone decalcification the calcium would be free to enter the serum and produce hypercalcemia

Follis We must get this straight We do not think that the presence of osteoid indicates that something has been withdrawn Instead we believe that something has not been deposited in the matrix which has been formed

THE RELATIONSHIP OF OTHER TUMORS AND RICKETS

One sees a similar picture in various tumors Figure 67 is from a car

Lawrence J H Tuttle L W Scott K G and Connor C L Studies on Neoplasms with Aid of Radioactive Phosphorus I The Total Phosphorus Metabolism of Normal and Leukemic Mice *J Clin Investigation* 19 267 (1940)

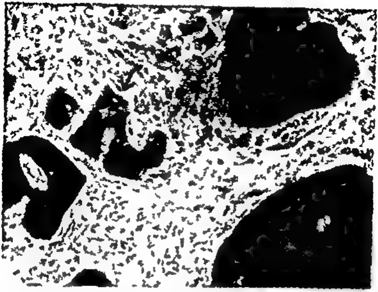

Fig 67 Osteoid (Non Calcified Matrix) in the Vertebral Body in the Region of Metastatic Carcinoma Cells (Breast)

The arrows indicate the osteoid

cinoma of the breast which has metastasized and the matrix which is formed in the presence of these tumor cells does not have inorganic material in it You could say as I think Dr McLean might say that this also is matrix which is not ready physiologically for the deposition of inorganic material This could be true but we cannot prove this one way or the other

Ernst It also may be that the matrix is incompletely calcified rather than uncalcified Are these sections of decalcified bone?

Follis Partially decalcified at least by our method We have controlled it with nondecalcified material Figure 67 indicates as far as silver nitrate goes a complete absence of calcification in an undecalcified section

Engel Dr Follis when you say that the matrix does not have the mineral in it do you mean that it does *not* contain calcium phosphate or do you mean that it is devoid of cations

Follis No! I do not think it is devoid of inorganic material but it is devoid of the normal complement of inorganic material which is necessary if you want to call it bone Cartilage which is uncalcified contains calcium

a d plo pl oru l t t relat ely lo v co m trat o t al o c ta s d and otl r organ c mater al Th s prol al l tr e of teo d too There are all degrees of morgan c impregnat on of cart la e and bone matr The el em t w ll tell you tl e opt n al asl content s—what s it —about 65 per cent?

Kra er Yes

C ina Tl at s ratl er an portat to t Dr Pappe l er an l l s a o tes some ea s ago l stud es on tl e re ult n of rats g e para tl yro l l or one a l re al le to sho v p t c l ml l re l t s e a al n an l by n cro nc ne at on tecl q es tl t the calc n co tent of tl e a ed k lney may be ery cons deral l n reased l efore tl ca l e demonstrate l l y ord ary ta n ng metl od o l x ra Tl e n pl cat on s that n cl calc um may be pre ent m tl e f rm of so e n on zel calc prote n or otl er con plex or wha e er ou n av a t to call t a l not n a f m wl cl takes the sl er ta n or can l e v al zed ro ntgenogran Tl e ab ence of calc um l y tl e ta n tecl que wl l e ord ar l regar l a n l a ng the presence of calc m (Dr Follis correctl po nts out that the e are reall phospl ate ta ns) loes not mean that no alc m pre ent

Follis I agree perfectl Sl er ta t n er tl at tl ere e n h norga c n ater al to g v a re a to th tl e ta n It f rtu e tl at o ca n o ta n osteo d t a al ze Ho e er n an get cart l ge a l t l a f n l w n ca tl c son e years a tl at o c l cr e a n ea g c en rat o of calc u an l pl o pl oru a o a r a l tl e area of pro o al c l fi at on

The Pathogenes s of Oste t s Fibrosa

TABLE XXVII

The Patl o ne s of O te t Fibrosa

1) Ac t se ere n t on
) Ac d s (local)
3) Pr n ar l perparatl r d sn
4) Secon lar l perparatl r l s
   (V a on n erum cal an l/or pl o pl r)
5 H pertl ro d
( ) Paget s d ea e
7) T or cell n marro ca tv
   (M elom a leuken a car no m and o fortl)

D ue W Sp ga n C and Pappen ei A M The Ca u n Con en of e k d e a Rela ed o Pa a h od Func on, *J Exp* *Med* 66 607 (1937)

Fig 69 Osteitis Fibrosa from a 17 Year Old Adolescent Child with Chronic Renal Insufficiency

This is an instance of practically pure osteitis fibrosa without excessive osteoid

Follis Yes some can be noted in Figure 72B

Shorr Are the osteoblasts common or rare?

Follis Oh they are fairly common This condition is fairly easy to detect We now have some 20 cases that we have studied at autopsy most of them in the pre thouracil period

Shorr What is the ratio of the first type with osteoporosis to the total incidence of bone involvement?

Follis Well a number of x ray studies have been carried out in which I do not think it was more than 10 per cent even less

Shorr No I meant in your pathological material

Follis I think that less than half of them had osteoporosis

Shorr And not the picture of Figure 72A?

Follis The illustration I showed in Figure 72A of the excessive destruction was from our worst case but we have many others like it though not

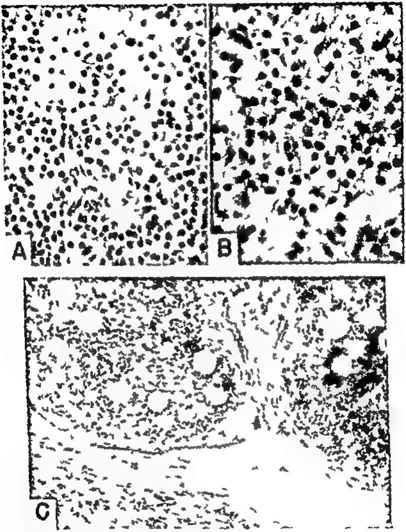

Fig. 70. Chronic Renal Disease in an Adult

A—Normal parathyroid gland of an adult. *B*—Parathyroid gland (same magnification as in A) of an adult dying of chronic glomerular nephritis to be compared with that in A. Note the clear cells in section *B*. C—Vertebral bone from the same patient showing osteitis fibrosa.

so extreme. Almost every case of active hyperthyroidism shows evidence of increased destruction of bone.

Shorr: What I meant was this: you showed two typical pictures, one

was the picture of osteoporosis and the other was the picture of osteo fibrosis. Which is more common?

Follis: Osteitis fibrosa, that is, under the microscope. These individuals do have skeletal manifestations clinically. They may complain of back pain; they may have spontaneous fractures. It is probably well known to you that von Recklinghausen in his monograph[23] described osteomalacia in a case of Basedow's disease, so this bone disorder has been known for sixty years. There is very little concerning the histological changes in the American literature, however. One also sees in some of these cases, Dr. Shorr, evidence of osteomalacia, as you might expect.

Robinson: Is it typical to have the areas of bone resorption in the center of the trabeculae?

Follis: You see this in primary hyperparathyroidism. The areas are not necessarily always in the center, but the cases of secondary hyperparathyroidism which I showed you had the areas of resorption in the center of the trabeculae as the result of renal disease. These are just manifestations of extreme abnormalities in calcium and phosphorus metabolism. Unfortunately, we do not have any determinations in our thyroid series of the levels of calcium and phosphorus in the serum. In over 20 cases we have only one x-ray, in which it is interesting that leukemia was considered because the bones were rarefied.

Reifenstein: Dr. Follis, is there any difference in the appearance of the parathyroid glands in these two different types of hyperthyroidism, the one with bone destruction and the one with osteoporosis as the predominant lesion?

Follis: Unfortunately, we do not have enough parathyroid glands to study, so I cannot answer your question.

Albright: How about the increase in osteoid on a physiological basis? Professor J. Erdheim used to mention two causes for wide osteoid strips on the surfaces of the trabeculae: 1) failure of calcium to be deposited in the osteoid and 2) such rapid osteoid formation that calcification does not keep quite abreast of it. One sees the latter in osteitis fibrosa generalisata whether due to hyperparathyroidism or renal insufficiency.

Follis: You should not see osteoid at this age; you really should not see any osteoid in an adult 30 or 40 years old. In our control cases (people who have died in accidents and so forth), you do not encounter this amount of osteoid. You do not see it in all cases of hyperthyroidism, Dr. Albright.

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23 Recklinghausen, F. v.: Fibrosis or Deforming Osteitis, Osteomalacia and Osteoplastic Carcinomatosis in their Mutual Relations. *Festschr. R. Virchow* Berlin (1891)

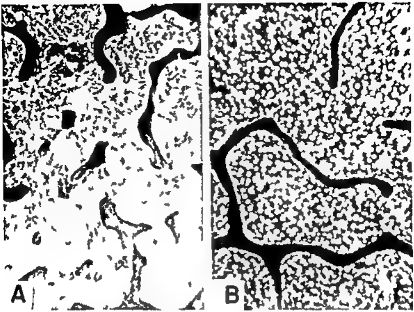

Fig 71 ' Osteoporosis and Hyperthyroidism

1 No n al vertebra of an adult *B*—Ve tebra ith osteoporos s (same magn f on) f an ad lt dy ng th hyperthyro d sm

THE RELATIONSHIP OF PAGET S DISEASE AND OSTEITIS FIBROSA

Anotl er situation in wh ch there is excessive destruction of bo e is Paget disea e (osteitis deformans) I think Dr Albright an l Dr Rei fen tein have justification for saying that Paget s disea e is primarily a lisea e of l one destruction Figure 73A is a very early instance You coul l n t determ ne from th ection whether it is Paget s d ease or ju t l ttle lacunae w th osteol lasts from on e other cause You have to exam ne more a lvance l cases in wh ch tl e evi lence is very much more marked h stol gically for example uch as in Figure 73B in which there is ex ce s e dens ty

THE RELATIONSHIP OF MULTIPLE MYELOMA AND OSTEITIS FIBROSA

Another interestin g question is thi How is bone destroyed when exoge

[1]Re fen te n F C J and Albr ght F P get s D sea e It Pathol g c Phys ol gy and the Importance of Th s in the Compl cat ons A ng from Fract e a d Im ob l z t on Ver *L J a I J Med* 231 43 (1944)

Fig. 72. Osteitis Fibrosa and Hyperthyroidism

A—Bone section with extreme osteitis fibrosa in an individual dying with severe hyperthyroidism. B—Higher power magnification of A showing osteoclastic reaction.

nous or new cells are found in the marrow? Figure 74 is an instance of multiple myeloma of the plasma cell type. There does seem to be evidence of active destruction; in other words, there are osteoclasts with connective tissue for some reason. It looks as though the bone is being eaten away. I do not see how you can say that there is pressure inside the bone. The tissue is as loose as you could want, and in the bone the plasma cells do not appear to be producing pressure. What they are doing to the nutrition of the bone, as in the case of leukemia, we do not know.

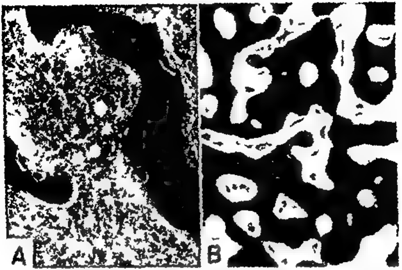

Fig 73 Osteitis Fibrosa and Hyperthyroidism

A—Bone section from early stage of Paget's disease showing beginning destruction of bone trabecula *B*—Bone section from late stage of Paget's disease showing marked increase in number and size of trabeculae

In myeloma again we see osteoid borders We do not see them in all of the cases and unfortunately we do not have serum calcium and phosphorous determinations in all of our cases so that we have no way of correlating the presence or absence of these osteoid borders in multiple myeloma with the mineral level in the serum

Howard Is that with or without renal insufficiency? Does it have any connection with renal insufficiency

Follis There does not seem to be detectable renal insufficiency—and by detectable I mean any clinical or other evidence

THE RELATIONSHIP OF SARCOIDOSIS AND OSTEITIS FIBROSA

There is one other condition I wish to discuss sarcoidosis I hope there will be some comment about it We do not know where to place sarcoidosis and it was not included in either of the two classifications which we have presented In a certain percentage of cases of sarcoidosis there is x-ray evidence of bone disease in the form of little areas of destruction particularly in the hands the fingers and the toes

Fig 74 Osteitis Fibrosa and Multiple Myeloma

Note the area resembling osteitis fibrosa along the edge of the trabecula which appears to indicate active bone destruction

In a series of 48 cases of sarcoidosis which we have confirmed histologically we found that there were 32 whose extremities had been x rayed In these 32 only 4 showed x ray lesions which we accept as typical of sarcoidosis However in sarcoidosis there are disturbances of calcium and phosphorus metabolism (calcium particularly) Of these 48 cases of sarcoidosis that were proved histologically 6 had serum calcium values above 11 5 mg per 100 cc Most of the cases that had hypercalcemia showed evidence of calcification of the kidneys by x ray However there was no evidence of involvement of bone by x ray That makes the picture even more complicated What is going on in sarcoidosis what role the skeleton plays in the hypercalcemia to me at least is not very clear One would expect that the hypercalcemia represented excessive bone destruction but you cannot find the evidence for this In 15 autopsied cases we have found only one in which there was extensive sarcoid tissue in the skeleton Unfortunately as far as I know no investigator ever has examined the lesions of the hand and feet in sarcoidosis histologically to determine whether sarcoid tissue is producing the changes

This is all I have to say Dr Armstrong I am sorry that it has taken so long

THE PROBLEM OF PARATHYROID ACTIVITY IN THE FIRST YEAR OR SO OF LIFE

ROBERT A. McCANCE

From the Department of Experimental Medicine
University of Cambridge, Cambridge, England

Armstrong: Dr. McCance is going to continue our discussion. I would like to have him tell you about a new disease occurring in infants affecting the parathyroid glands.

Evidence for Decreased Parathyroid Function at Birth

McCance: There are observations from a number of sources that suggest that some aspects of parathyroid activity are low at and soon after birth. We have the absence or virtual absence of phosphate from the urine of the newborn [1] and the ease with which the serum phosphate level can be raised by the administration of phosphates.[1] Evidence of a similar nature has been collected by other investigators interested in tetany of the newborn.[2-4] The fall in the serum calcium level, which is thought to be the cause of the neurological hyper excitability, is explained by most investigators as secondary to the rise in the serum phosphate level which itself is believed to be due to an intake, generally from cow's milk, greater than can be deposited in the bones or eliminated through the kidney when parathyroid function is negligible.

There appears to be evidence [5] that in these cases with a high serum phosphorus level the parathyroids show histological signs of over activity, and this has been regarded as an attempt on the part of these glands to produce sufficient functional activity to maintain the serum phosphorus

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[1] McCance, R. A., and [illegible] M. A. [illegible]: The Titratable Acidity, pH, Ammonia and Phosphates in the Urine of Very Young Infants. *Arch. Dis. Child.* [illegible] 200 (1948).

[2] Bakwin, H.: Pathogenesis of Tetany of New Born. *Am. J. Dis. Child.* 54: 1211 (193[illegible]).

[3] Gardner, L. I., MacLachlan, E. A., Pick, W., Terry, M. L., and Butler, A. M.: Etiologic Factors in Tetany of Newly Born Infants. *Pediatrics* [illegible] 228 (1950).

[4] Gittleman, I. F., and Pincus, J. B.: Influence of Diet on the Occurrence of Hyperphosphatemia and Hypocalcemia in the Newborn Infant. *Pediatrics* 8: [illegible]9 (1951).

[5] Gardner, L. I.: Tetany and Parathyroid Hyperplasia in the Newborn Infant: Influence of Dietary Phosphate Load. *Pediatrics* 9: 534 (195[illegible]).

concentration within normal limits Snelling[37] has suggested that th rise in serum phosphorus level soon after birth may be due in some cases to a disturbance in renal function associated with nitrogen retention and not therefore to parathyroid under activity

If we consider the serum calcium level in the newborn there is normally according to Bakwin[233] a fall soon after birth from the high levels usually found in cord serum This is attributed to the rise in the serum phosphoru concentration which usually takes place about the same time but in adul hypoparathyroidism hyperphosphatemia is not always the rule although hypocalcemia is one of the most constant and characteristic features of this condition Except during the first few days of life the serum calcium leve of the child is not normally below that of adults yet the serum phosphorus is usually above the adult level Are we to suppose that the parathyroids are at this time functioning as they do in adult life?

A Syndrome Hypercalcemia in the Infant

I have presented these facts and considerations as a background to the interesting cases of pathologically high serum calcium values observed in the first year of life A number of these patients have been described in England recently by Lightwood of St Mary s Hospital and by Payne of Great Ormond Street Hospital and we have ourselves had the chance of investigating a case of Dr Gairdner s at Cambridge Although I believe that Lightwood recognized the nature of these cases without help from the literature I feel certain that the syndrome has been described before e g by Pratt Geren and Neuhauser and by Chown [9] I suspect more over that the cases of chronic hypercalcemia raised blood level mental and physical retardation and signs of osteosclerosis should be grouped with this syndrome but these cases are certainly more complicated and show differences as well as similarities [0]

The clinical features of our own case which is I believe a fairly typical one have been as follows

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[37] Snelling C E Disturbed Kidney Function in the Newborn Infant Associated with a Decreased Calcium Phosphorus Ratio *J Pediat* 22 550 (1943)

[?] Pratt E L Geren B B and Neuhauser E B D Hypercalcemia and Idiopathic Hyperplasia of the Parathyroid Gland in an Infant *J Pediat* 30 388 (1947)

[?] Chown B Renal Rickets and Dwarfism a Pituitary Disease *Brit J Surg* 23 552 (1936)

[240] Fanconi G Girardet P Schlesinger B Butler N and Black J Chronic Hyperglycemia Combined with Osteosclerosis Hyperazotemia Nanism and Congenital Malformations *Helvet Paediat Acta* 7 314 (1952)

The ch ll aged 8 m nth wl o or l mally has a n rmal tw r s ter was brought to t e hosp tal t November 1951 l ecause she was ot thr v ng and v as always prone to v m t ng Her we ght was 15 lb S e was found to h ve a mod erate degree of anem a a small quant ty of pus n her ur ne a d a sl ght fever The e ch ldren, it seems always t nd to be const pated a d th s one was n excep t on to the ru e On adm ss on a cl n cal d agnos s of renal ac d s s as enterta ned but t e b ochem al f nd gs fa led to confirm th s m press on (Table XXVIII) Wh le th pat e t wa the h sp t l he was treated w th chlor myc t n and sulfamethaz ne but after d charge l e cont nued t l a p ur a

TABLE XXVIII

Patient K I with Hypercal em a The Findings in the Blood Serum and in the Urine in November 1951

Blood Serum	
Carb n d x de c mb n ng p wer	24 3 m q / l
Chlor de (pla ma)	107 m q / l
Urea	42 mg 100 cc
Urine	
pH	6 1
Specific gr vity after 6 hours without fluid	1 015

On th 24th of Apr l 1952 the bl od urea was f und t have ris n to 63 mg per 100 cc Fol w ng L ghtwood s report on h s cases at t e W ndermere meet ng of the Brit h Ped atric Associat n May t e serum calcium level of the ch ld wa det rm ned a d as f und to be 15 3 mg 100 cc and at th s t me the nor gan c phosph rus level was 4 2 mg 100 the n n prote n n trogen concentration was 55 mg /100 cc and the total prote n level wa 7 4 gm 100 cc The electro- phoret c pattern f the serum prote n was w th n normal l m t O the 31st of Jul her we ght wa st ll 15 lb 4 z The ur ne was ster le n l c nt ned a smal am unt f pu as before

In Augu t 1952 a balance t dy was carried ut with great d ff culty (Table XXIX) and the f n ings in the bl od the cerebr sp nal fluid and

TABLE XXIX

Patient K I w th Hypercalcemia The Mineral Balances in August 1952

Mineral	Intake	Excretion			Balance
		Urine	Feces	Total	
	mg 48 h	mg / 48 h	mg 48 h	mg / 48 h	mg / 48 h
Calcium	1140	104	471	575	+565
Magne ium	133	25	73	98	+35
Phosphoru	715	297	185	482	+233

the urine at that time are given in Table XXX. The child was placed on a very low calcium diet which also contained only a small quantity of phosphorus and consisted mostly of orange juice (the only nourishment she would take apart from milk); after four days the observations shown in Table XXXI were made. Attention is called to the small fall in the serum calcium level which may not be significant, to the fall in the phosphorus excretion, and to the absence of any other noteworthy change. These findings suggest to me the presence of a normal mechanism for the parathyroid control of the phosphate excretion and also perhaps of the serum calcium level.

A moderately low calcium diet was instituted on the 20th of September. Tube feeding was tried in order to maintain the calorie intake but this had to be abandoned. The calcium in the food was derived almost exclusively from milk and amounted to 200 to 300 mg. per day. On this treatment the child improved clinically and became much brighter but her appetite did not improve. On the 8th of

TABLE XXX

Patient K. P. with Hypercalcemia. The Findings in the Blood Serum, the Cerebrospinal Fluid and the Urine on August 19, 1952

Blood Serum	
Calcium	16.5 *mg./100 cc.*
Magnesium	3.02 *mg./100 cc.*
Phosphorus	5.2 *mg./100 cc.*
Urea	54.0 *mg./100 cc.*
Alkaline phosphatase	4.0* *Bodansky units*
Serum protein electrophoretic pattern	Within normal limits
Cerebrospinal Fluid	
Calcium	6.4 *mg./100 cc.*
Magnesium	3.18 *mg./100 cc.*
Urine	
Citric Acid†	
Specimen I	11‡ *mg./100 cc.*
Specimen II	14‡ *mg./100 cc.*

*Normal level for infants 5 to 13 *Bodansky units*.

†Determined by the method of Hargreaves, Abrahams and Vickery[44] in two 24 hour specimens.

‡Within the normal range for adults.

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[44]Hargreaves, C. A. II, Abrahams, M. III, and Vickery, H. B.: Determination of Citric and d-Isocitric Acids. *Analyt. Chem.* 23: 467 (1951).

TABLE XXXI

Patient K P with Hypercalcemia

A The Findings in the Blood Serum and in the Urine after Four Days on a Very Low Calcium Diet on September 5 1952

Blood Serum	
Calcium	*14 3 mg /100 cc*
Phosphorus	*5 2 mg /100 cc*
Alkaline phosphatase	*3 1 Bodansky units*
Total protein	*7 2 gm /100 cc*
Albumen	*5 1 gm /100 cc*
Globulin	*2 1 gm /100 cc*
Urine	
Calcium	*109 mg /24 hr*
Magnesium	*16 mg /24 hr*
Phosphorus	*148 mg /24 hr*

B The Findings in the Blood Serum on October 22 1952

Blood Serum	
Citrate	*2 3* mg /100 cc*
Total calcium	*13 7 mg /100 cc*
Ultrafilterable calcium	*7 7 mg /100 cc*

* Well within the normal range for adults

October 1952 her serum calcium level was 14 4 mg per 100 cc her blood urea concentration was 37 mg per 100 cc and her alkaline phosphatase level was 2 2 Bodansky units

Evidence for Increased Parathyroid Function in the Syndrome

Most of the English cases have recovered spontaneously and our patient is still alive so I can present no postmortem evidence as to the state of the parathyroids Pratt and his associates [8] found histologic signs of parathyroid over activity in their case but Chown made a careful search and could find no evidence for it the parathyroids in his case were not hypertrophied Fanconi and his associates [9] removed two parathyroids from their case by operation The glands were about 2 mm in diameter the histologic examination revealed a large number of almost water clear cells which the authors regarded as a sign of hypertrophy but the removal of the gland made little difference to the clinical progress of the patient and I feel

the interpretation of the histologic findings must be accepted with some reserve Most pathologists I suspect know very little after all about the appearance of normal parathyroid glands at this age

Should we regard these cases as disorders resulting from parathyroid gland overactivity with secondary but usually slight renal involvement? With such a high serum calcium level one would expect to find in adults a very low serum phosphorus level and I find it difficult to accept this degree of renal failure—as evidenced by the blood urea concentration—as enough to prevent a fall in serum phosphate level if the parathyroids were over active Furthermore Fanconi and his associates [40] gave 25 units of parathyroid extract to their patients and obtained an immediate fall in the concentration of phosphorus in the serum a rise in the excretion of phosphorus in the urine and a further increase in the concentration of calcium in the serum We have repeated this procedure and have obtained similar result for the serum but no significant change in the hourly output of phosphorus in the urine (Table XXXII) I believe that Dr Payne and Dr Lightwood have administered parathyroid extract and have observed responses similar to those in the Fanconi case [2,6] If the parathyroids are over active one would expect in some of these cases to have found some evidence of this in the bones and hence in the plasma alkaline phosphatase level unless the parathyroid hormone does not produce lesions in the bones at this age or in children with a sufficiently high calcium intake

Possible Mechanisms of Pathologic Physiology for the Syndrome

The whole question to me seems very obscure (Table XXXIII) I feel we can rule out over activity of the parathyroids due to a tumor but not functional over activity of a normal gland The workers at Great Ormond Street Hospital and at St Mary s Hospital tend to regard these patients as children who (for some reason or other) are abnormally sensitive to vitamin D but this is a supposition and so far without any proof

We also have considered whether or not these children should be classed with the adults who have been shown to have a somewhat similar clinical picture after an excessive intake of milk [24, 43] The milk drinkers also have generally been persons who have taken very large quantities of alkali Whether this ingestion of alkali has contributed to their trouble I do not

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Albright F and Reifenstein E C Jr *The Parathyroid Glands and Metabolic Bone Disease Selected Studies* Balliere Tyndall and Cox London Williams and Wilkins Co Baltimore (1948)

[43] McQueen E G Calcium Gout and Milk Poisoning *Lancet* 11 67 (1952)

TABLE XXXII

Patient K P with Hypercalcemia The Effect of Parathyroid Extract on the Urinary Excretion of Calcium Phosphorus and Magnesium and on the Blood Serum Levels of Calcium and Phosphorus on October 22 1952

Time	Urine Volume	Urinary Excretion: Calcium per 100 cc	Calcium per mg of creatinine	Phosphorus per 100 cc	Phosphorus per mg of creatinine	Magnesium per 100 cc	Magnesium per mg of creatinine	Blood Serum: Calcium	Blood Serum: Phosphorus
	(cc)	(mg)	(mg)	(mg)	(mg)	(mg)	(mg)	(mg/100 cc)	(mg/100 cc)
9 a m	58.5	7.9	1.4	17.4	3.0	1.85	0.33	—	—
10 a m	42.0	9.5	1.0	20.4	2.1	2.70	0.25	13.7*	4.65
Parathyroid Extract 25 Units (1.25 cc) Intravenously at 10 a m									
11 a m	58.0	5.2	1.5	17.6	4.5	0.2	0.13	—	—
12 noon	46.0								
1 p m	20.0	6.0	1.5	25.5	6.3	1.60	0.40	—	—
2 p m	68.0	5.0	2.5	14.0	7.0	0.94	0.47	—	—
3 p m	30.0	5.0	2.0	20.5	9.6	0.90	0.37	—	—
5 p m	33.0	10.9	1.7	26.0	4.0	1.66	0.26	—	—
6 p m	26.0	7.7	—	15.4	—	1.27	—	16.2	3.5

Ultrafilterable calcium mg /100 cc

TABLE XXXIII

Patient K P with Hypercalcemia Possible Mechanisms

1 Relea e of too much parathyroid hormone due to a) a tumor b) functional overactivity of a normal gland
2 Abnormal sensitivity to vitamin D
3 Excessive absorption of calcium
4 Renal failure involving retention of calcium a) because the kidney for some reason reabsorbs too much calcium from the glomerular filtrate b) becau e the kidney is unable to maintain the serum phosphorus level within normal limits, hence the serum calcium level falls and the parathyroids o er react
5 Abnormal metabolism of citrate or other substance which might interfere with calcium metabolism

know but the children certainly have not been over dosed with alkali and (far from having high plasma bicarbonate levels) some of the Great Ormond Street patients have had renal acidosis with therefore very low figures for their plasma bicarbonate concentration

We have considered also whether or not the primary lesion could be in the kidney but if so it must be a specific one leading to the reabsorption of more than the usual amount of calcium Whether thi is the case or not *the output of calcium in the urine seems to* be quite normal in spite of the high serum calcium levels for children of this age on their usual intakes of calcium and hence positive balances are to be expected

Finally we have considered whether or not the abnormal metabolism of citrate or some other such sub tance might be the underlying cause but we have not thus far found any evidence for this Some clinicians with whom I have discussed this question are satisfied by the idea that a biological steady state has been established in these children at an abnormal level of serum calcium This explains the clinical findings in a way but does not constitute a diagnosis as I interpret the term

Parathyroid function in early life seems to be a problem which will require a good deal more investigation before we can understand it and fit it into its proper place in infant physiology

Conference Discussion

Armstrong: Thank you, Dr. McCance.

Follis: Have these cases in England had the other characteristics of the syndrome that Fanconi has described,[10] that is, have they had all of the abnormalities?

McCance: No. The case that Schlesinger described—

Follis: That would be Fanconi's Syndrome No. 2, I guess.

McCance: I am talking about the cases described by Fanconi, Girardet, Schlesinger, Butler, and Black.[2,8]

Shorr: Dr. McCance, how old are these children?

McCance: In the first year of life. Our child was eight months old and I think that is fairly typical.

Shorr: I am just wondering whether the problem of renal development in children might have something to contribute. I understand that the kidney in the infant is far from being a kidney capable of taking load, and that there has to be progressive development, that is, actual morphologic as well as functional development. Is that correct?

McCance: I know something about the kidney in infancy, but I do not know anything about it which will explain these findings.

Kramer: How about the glomerular filtration and reabsorption of phosphorus as a possible indication of parathyroid over-activity?

Butler: I would like to comment on that, too. If you are trying to decide whether or not this is excessive parathyroid activity, then the way to do that is to determine what Crawford and Talbot[*] call *The Parathyroid Index*, namely, the ratio of the tubular reabsorbed phosphorus to the glomerular filtered phosphorus. That would give you, I think, a correct appraisal of the parathyroid function of your patient. Then I should think you should use such a measure of renal function as the urea clearance.

McCance: We have done the urea clearances.

Butler: And they were what?

McCance: They were below normal when the serum urea level was raised.

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[*] Crawford, J. D., Osborne, M. M., Jr., Talbot, N. B., Terry, M. L., and Morrill, M. F.: The parathyroid glands and phosphorus homeostasis. J. Clin. Invest. 29: 1448-1461 (1950).

Handler When you have indications of renal insufficiency of moderate degree I do not think you should rule out hyperparathyroidism The fact that the phosphorus concentration is normal is an indication that the patient is still sufficiently able to respond to parathyroid hormone which has lowered what otherwise would be an elevated plasma phosphate concentration to a normal level simply raising the calcium concentration

Kramer Although I do not know about children of this age in very young infant the concentration of serum inorganic phosphorus is higher than the values you have given and what you have in this patient may represent a substantial decrease below the normal

Butler I would doubt that it should be higher at this age at eight months I think a serum inorganic phosphorus level of 5 mg per 100 cc is perfectly normal The level is elevated to three weeks of life and then elevated only if you are giving a diet that is excessively high in phosphate

McCance In reply to your question Dr Handler I think that the loss of renal function in this patient is secondary to the rise in the serum calcium because in some cases which Lightwood has observed there has been calcification of the kidneys and the renal function tends to return to normal when the serum calcium falls I would regard the retention of nitrogen and the abnormal renal function here as being of the type found in vitamin D over activity

Shorr Does the renal function return to normal if there has been renal calcinosis or only in those cases in which there has been no renal calcinosis?

McCance I cannot tell you

Shorr One would wish that that were the inevitable outcome of renal calcinosis

McCance There is generally only a mild degree of renal calcinosis in these cases

Shorr And how demonstrated therefore?

McCance I think radiologically

Shorr A mild degree demonstrated radiologically?

McCance Well we may differ in our assessment of mild and severe I can speak only of our own case in detail and our case has not shown any sign of renal calcinosis radiologically

Harrison The urinary citrate studies interest me In hypercalcemia due to vitamin D poisoning the serum citrate level is high and the urinary citrate excretion is increased The same finding is seen also in hypercal

cemia due to hyperparathyroidism as was pointed out by Dr Shorr and as we found also in a few cases In this patient the urinary citrate excretion is not increased and actually the values of 10 to 14 mg per 100 cc are on the low side of normal

McCance We were using the method of Hargreaves Abrahams and Vickery [1] and we were getting good recoveries

Harrison The values are in the low normal range on an ordinary milk diet The urinary citrate excretion does not fit in with our limited experience in vitamin D poisoning or hyperparathyroidism It would be interesting to know whether or not the calcium is bound in some unusual fashion so that it is partitioned in an abnormal manner between the protein bound and the ultrafiltrable portions

McCance We did measure the ultrafilterable calcium fraction too and of course it was raised with the total serum calcium level but the proportion of ultrafilterable calcium was similar to that found in normal adults

Harrison How was the ultrafiltration done Dr McCance

McCance We did it in a small collodion sac under pressure by the method described by Greenberg and Gunther We equilibrated under CO_2 and oxygen

Armstrong Dr Howard has some cases which will add to the discussion

Howard I do not think that they will although I had hoped the problem would be brought up This syndrome which you have described is significant I think in relation to one of the points which I mentioned in the discussion and which I hoped would call forth comment This is the problem posed by individuals who have hypercalcemia and normal serum phosphate levels who obviously do not have an absorptive increase to explain the hypercalcemia because when you eliminate the calcium in the intake and feed them otherwise a perfectly normal diet the serum calcium level does not fall appreciably The cases that we have seen in whom this situation exists are five patients with cancer of the lung two of whom had no evidence at postmortem or by x ray of skeletal metastases or rarefaction and in whom the serum phosphatase level was perfectly normal We have observed I should say at least half a dozen patients with sarcoidosis with this same syndrome

I saw one of these infants that you are describing at Great Ormond Street Hospital two years ago The professor of pediatrics at Guy s Hos

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Greenberg D M and Gunther L On the Determination of Diffusible and Non Diffusible Serum Calcium *J Biol Chem* 8 491 (1930)

pital took me around and showed me a baby whose serum calcium was 16 mg per 100 cc and who had all of the other manifestations that you have described The spinal fluid calcium of this child was measured at my suggestion and it turned out to be quite high so that hyperparathyroidism seemed rather unlikely But I do not think that you could rule out the existence of a parathyroid adenoma because we have seen infarcted parathyroid adenomas which turned themselves off and temporarily at least completely recovered

I do not see where the calcium comes from in hypercalcemia sarcoidosis as I said in my previous remarks it seems to me that it must come from the bone There must be a situation locally at the skeletal level which keeps the hypercalcemia going because when you eliminate all of the dietary calcium the serum calcium level does not fall and the hypercalciuria continues bone is the only source I can think of that could support the abnormal serum and urinary calcium values for any long period of time Some of the sarcoidosis cases we have seen have had renal calcinosis and others have had renal insufficiency but without calcinosis even at post mortem Dr Follis can tell you about that

Follis There have been several cases of chronic glomerular nephritis associated with sarcoidosis

Howard Well chronic glomerular nephritis is more common in sarcoidosis than it should be in the population at large In the patient at Great Ormond Street Hospital I suggested the diagnosis of sarcoidosis and Dr Philip Evans said there was no evidence for it but he had not done any tuberculin test at that time If that was not negative I suppose you could rule sarcoidosis out But the hypercalcemia of sarcoidosis does turn itself on and off spontaneously You may see a person with a normal serum protein level and a calcium value elevated to 15 mg per 100 cc then he will suddenly come back with the sarcoidosis apparently having stopped whatever it was doing and the serum calcium level will be normal for a time One of the most dramatic effects that cortisone has is to bring the serum calcium level down very promptly to normal in patients with sarcoidosis The response may last for two weeks or it may last for eight months with a single course of cortisone How the cortisone works I do not have the slightest idea but it certainly does work for we have seen it over and over again

Follis Then we have studied at least one case in which previously there must have been a sarcoidosis because we found the scars of the sarcoid with the Schaumann s bodies and we assumed that probably there had been a preceding hypercalcemia with renal insufficiency The sarcoidosis healed and the individual went on to develop secondary hyperparathyroidism

Fremont Smith Is the cerebrospinal fluid serum calcium elevated in sarcoidosis?

Howard When the serum calcium is At least we have seen it in our cases

Fremont Smith So that sets this disorder apart from hyperparathyroidism?

Howard Well unfortunately the law of hyperparathyroidism hypercalcemia cases having a normal spinal fluid calcium level was broken by Dr Charles Dent during this past year when he found a patient with proven hyperparathyroidism in whom the spinal fluid calcium level was elevated

Fremont Smith But a single case—

Howard This is the only one I know about None of our patients has had this finding

Butler Dr McCance why do you say that the cases such as the one you have described resemble those with acidosis?

McCance Clinically they are indistinguishable

Harrison Do they have polyuria?

McCance Yes I think that they do We measured the calcium level in the cerebrospinal fluid in this case and it was 6 4 mg per 100 cc when the serum calcium concentration was 16 5 mg per 100 cc In regard to Dr Howard s question of where the calcium in the serum comes from we must remember that we did find very large positive balances If these balances are correct there is plenty of calcium being absorbed to maintain the level in the serum

Howard That may be This brings up another question why do parathyroid adenomas often produce no rarefaction although we know that they have been present and causing hypercalcemia and hypercalciuria for years Is this a form of adaptation?

But let us return to the syndrome that you have presented Dr McCance I think Dr Philip Evans did this experiment for me while I was at the Great Ormond Street Hospital but I will not swear to it from memory As I recall it Dr Evans placed the patient for a matter of some days on a practically zero calcium intake (These patients are a feeding problem anyway) The patient was a five year old incidentally who evidently had had the calcium disturbance intermittently every time something happened and who had had meningitis too I might say The calcium in the serum remained elevated (this may be a protective mechanism) therefore dietary calcium certainly is not the primary source of the hypercalcemia

McCance Don't you think it is rather curious in the case I reported that there should be so little calcium in the urine in spite of the very high serum calcium level?

Stearns The urinary calcium is about three times that found in the urine of the normal infant of this age

McCance It is? I thought that it was within the normal range

Butler The serum level is about what you find in hyperparathyroidism

McCance The clearance would be very low

Butler The excretion may be high but the clearance low

McCance We must certainly determine Talbot's ratio I was not aware of the importance of it until I came over here and discussed the case with Dr Talbot I had not had the opportunity to read his book as it had not reached England when I left

Butler I would like to know whether this youngster was brought up on breast milk or on cow's milk in the first months of life

McCance On cow's milk

Butler The reason that I ask this question is because feeding an infant in the first few weeks of life a cow's milk formula of average strength causes increased function of the parathyroids That might start a chain of events which could lead to persisting parathyroid over activity

McCance Yes

Stearns Dr Butler we have studied some 300 odd babies fed cow's milk without ever running into the increased serum calcium level after the first ten days

Butler I agree You will not find an increase in the serum calcium level perhaps because the serum inorganic phosphorus level is increased but you may get hypertrophy of the parathyroid glands

Stearns I do not believe that the feeding of cow's milk *per se* could start such a train of events as this child has shown or we would observe it very frequently in American children

Butler If you look at the parathyroid glands during autopsies of babies fed breast milk and of babies fed cow's milk in the neonatal period you can tell which baby has been fed which type of milk We assume that the effect on the parathyroids is relatively transient We know that after the child is about two weeks of age the parathyroids can accomplish the excre

tion of the large amounts of phosphate that are being ingested. Whether in some children this hyperfunction would start a chain of events that might result in transient but persisting parathyroid over activity I would have no idea.

Stearns: But you would not expect to find excellent bone mineralization in an infant with definite hyperparathyroidism.

Butler: You might if you were having a large intake of calcium. The infant described by Dr. McCance was given a large intake, 1 point something grams of calcium a day. For an infant, that is, I would say a high calcium intake.

Stearns: These children show the same excretion on a low calcium intake.

Howard: Did any of those patients receive cortisone or ACTH, Dr. McCance?

McCance: No, I do not think that they did. We have been very conservative in our therapy because our evidence has indicated that these patients recover spontaneously, and we felt that the proper procedure was to let them do this if they could.

Howard: I would hesitate to give cortisone in the presence of renal insufficiency. Incidentally, I am not certain—how does the Talbot index work out in the presence of renal insufficiency?

Butler: In renal insufficiency you would have an end organ deficit that would mask the parathyroid function.

Howard: This individual obviously had renal insufficiency.

Butler: I would think that the patient had a very minor amount of renal insufficiency.

McCance: Yes, I think it was of a minor degree.

Howard: But the urea nitrogen ran around 50.

McCance: The blood urea, not the urinary urea nitrogen.

Howard: Oh, the blood urea! The patient of Dr. Philip Evans had blood urea values of 50 to 100 mg. per 100 cc., a much more serious renal defect.

McCance: These patients have had all variations and degrees of renal involvement.

Butler: If parathyroid hyperactivity persists, then that of itself can

produce the renal disease When you think of the number of children who have convulsions during the age period of six months to seven years and hence the number of times physicians measure the serum calcium and inorganic phosphorus levels then this type of patient must be very rare

McCance That may be true but if so it is remarkable how many have turned up in the last eighteen months in England

Armstrong Sarcoidosis has been mentioned several times not only in the opening presentation but also in the discussion of this syndrome I think Dr Gutman has some personal information on sarcoidosis is that correct Dr Gutman?

Gutman I have no data of my own on sarcoidosis other than the usual experience of encountering distinct hypercalcemia without enough x ray evidence of bone involvement to explain the elevation of serum calcium satisfactorily Dr Klatskin and Dr Gordon[146] of Yale recently have reviewed this whole subject but without being able to throw much more light on it They had two cases of sarcoidosis with hypercalcemia so closely simulating hyperparathyroidism that both patients were explored for parathyroid tumor none was found In neither instance was there impressive x ray evidence of skeletal decalcification The authors speculated about the origin of hypercalcemia in sarcoidosis and concluded that the excess calcium must be derived from the bones despite the paucity of evidence for this They pointed out that marked impairment of renal function may occur in this disease usually not due to sarcoid granulomas of the kidney (which are uncommon) but probably secondary to protracted hypercalcemia and sometimes associated with nephrocalcinosis and stone formation demonstrable in x rays They doubted that the renal lesions are primary or that the hypercalcemia is the result of impaired renal excretion of calcium

Engel Dr Follis could you say something about the pathology of sarcoidosis?

Follis I do not think that your question is germane to this discussion if you don t mind

Engel The reason I asked the question is that if connective tissue in general is involved this again might be an instance where its ability to bind calcium would be altered The calcium would then spill out into the serum

Follis I think that such an interpretation is completely incorrect because

[146]Klatskin, G and Gordon M Renal Complications of Sarcoidosis and Their Relationship to Hypercalcemia with a Report of Two Cases Simulating Hyperparathyroidism *Am J Med* in press

you see exactly the same reaction in generalized sarcoidosis as you do in generalized Hodgkin s and a variety of diseases As far as I know sarcoidosis is the only condition which is characterized by this peculiarity You see involvement of the bone marrow and of the bones in Hodgkin s disease and in virtually 100 per cent of the cases You do not see any abnormality of calcium metabolism of this degree in Hodgkin s disease

Howard We have one patient with Hodgkin s and hypercalcemia

Follis Oh yes we see an occasional one with a calcium disturbance but none that is as marked as in sarcoidosis

McCance Dr Park did you not say earlier that you had a case similar to the one I described?

Park I think the child was about ten years old Dr Albright remembers the patient very well indeed because we asked him to see the child and nothing abnormal ever was found except that the calcium was above 15 mg per 100 cc

McCance I did not understand that it was such an old child

Park Dr Albright was of the opinion that it was a case of hyperparathyroidism The child recovered completely

Howard Well clinically the same picture is produced and is reversible You may have it with extensive atrophy such as you see in polio

Those patients with sarcoidosis who have hypercalcemia with a normal protein level act exactly the same to ultrafiltration as those individuals in whom we produced the hypercalcemia by intravenous injection or by adding the calcium *in vitro* In those cases of sarcoidosis in which the levels of the serum protein and the serum calcium are elevated there is still an elevated ultrafilterable calcium

THE TRANSPORT OF CALCIUM IN PLASMA

ALEXANDER B. GUTMAN

From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and from the Department of Medicine, The Mount Sinai Hospital, New York, New York

Armstrong: I have prepared a list of names according to various diseases—I do not mean to say that you have the disease! By Dr. Gutman's name I have myeloma, hyperparathyroidism, and Paget's disease. Any comments on these?

Gutman: I have no comments on these topics, but I would like to provoke a little more discussion about the transport of calcium in the plasma. When Dr. Howard talked on this subject at the beginning of this conference, he directed most of his attention toward the ultrafilterable calcium moiety. Perhaps I could goad some of the physical chemists here into discussing more fully that fraction of the plasma calcium which is in combination with the plasma proteins.

The Nature of the Calcium Protein Complex

We usually speak of the protein-bound calcium in the plasma as if it were present as a single calcium proteinate complex. In all likelihood, however, there are quite as many undissociated calcium proteinates as there are discrete proteins in plasma, and at the last count I am aware of, there were at least 30 or 40 *different individual protein components* of the plasma, if one includes the various specific circulating antibodies. Each one of these proteins presumably has a specific chemical composition and presumably has its own calcium proteinate dissociation constant. The total plasma nondiffusible calcium moiety, therefore, should be regarded as the summation of a large number of different protein-calcium complexes which, if individually considered, would present a very complex situation indeed.

Armstrong: Dr. McLean, what is the name of the worker at Northwestern University who came out with about sixteen *different* dissociation constants and suggested that your $10^{-2.2}$ was an algebraic mean of these?

McLean: Irving Klotz.[24] He is in the Department of Chemistry.

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[24] Klotz, I. M.: The Application of the Law of Mass Action to Binding by Proteins. Interactions with Calcium. *Arch. Biochem.* 9: 109 (1946).

Howard Dr Gutman that is a well taken point and I did not want to go into that problem because we find it much too complicated

Gutman It is possible however to simplify the situation I believe by making certain broad generalizations

The Calcium Binding Properties of Plasma Protein Constituents

In normal subjects and in most diseases by far the largest proportion of the calcium bound to plasma proteins may be assumed to be bound to albumins since albumins ordinarily constitute some 55 to 60 per cent of the total plasma proteins and each gram of albumin apparently combines with something of the order of 0 8 mg of calcium when the total serum calcium is within the normal range however in disorders in which the serum albumin fraction is markedly reduced for example in the nephrotic syndrome this generalization presumably would not apply It also may be assumed that the proportion of protein bound calcium in combination with gamma globulins ordinarily is very small since the gamma globulin fraction normally constitutes only some 11 per cent of the total plasma proteins in man and the gamma globulins appear to bind little calcium something of the order of 0 1 to 0 2 mg of calcium per gram of gamma globulin In diseases in which there is very marked hypergammaglobulinemia however this may add up to a significant though still small figure The remainder of the non diffusible plasma calcium fraction some 1 to 2 mg in normal subjects (as measured by the usual ultrafiltration technique) presumably is bound to the alpha and beta globulins

Howard Dr Gutman where did you obtain these figures for the amount of calcium that is bound by each protein fraction of normal serum? Dr Hopkins of our group attempted to determine the calcium binding values of protein fractions that were sent to us by Dr Cohn We ran into the most enormous number of difficulties such as insolubility and how much lipoid mixture was included with these protein fractions Dr Cohn would send us a carefully labeled fraction one month and another the next month labeled exactly the same and we found totally different quantitative binding of calcium We became confused because some of the globulins even bound just as much as the albumin

The Calcium Protein Relationships in Serum

Gutman The figures that I cited we obtained ourselves by mathematical analysis of the relationship between the serum calcium content and the albumin and globulin content of some 160 sera obtained from patients with diseases associated with significantly increased or decreased plasma

protein levels unaccompanied by primary disturbances in calcium or phosphorus metabolism[248] These consisted chiefly of cases of the nephrotic syndrome lymphogranuloma venereum and hepatic cirrhosis

The regression equation which gave the best fit in these cases was

$$\text{Total calcium} = 0.85 \text{ albumin} + 0.2 \quad \text{globulin I} \quad + 7.0$$

In this equation *total calcium* is expressed in mg per 100 gm serum water The *quantity 0.85 is a constant indicating mg of calcium bound per gram of albumin* The quantity *0.2* is a constant representing mg of calcium bound per gram of *globulin* when the latter protein is present in excess of 3.0 gm of total globulins (i.e. this term is omitted as insignificant except in cases of marked hypergammaglobulinemia) The quantity *7.0* is a constant which is the sum of two constants *5.8 ± 0.2 mg of calcium per 100 gm* of serum water which represents the *diffusible (ionized) calcium* fraction that is assumed to be constant by definition (through exclusion of cases with a primary disorder of calcium metabolism) *plus* 1.0 ± 0.5 mg of calcium per 100 gm of serum water which represents what appears to be a relatively constant amount of *non diffusible (non ionized) calcium bound to alpha and beta globulins*

I hardly need to add that this type of analysis can give only the most gross of general approximations

More direct approaches to the problem together with the results obtained with various serum protein fractions were reviewed in 1944 by Greenberg[9] Relevant to this discussion is the estimate by Drinker Green and Hastings[250] *using the frog heart method for approximating the calcium* ion concentration of 0.0005 mM of calcium bound per gram of the horse euglobulin fraction P_{II} when $(CA^{++}) = 1$ mM per kilogram of H_2O This serum protein fraction subsequently was shown by Svensson[51] to consist electrophoretically of gamma globulins The very low figure for pK_{CaP} obtained for this horse serum gamma globulin fraction by Hastings and his associates is in accord with the deductions drawn from the study of human sera with marked hypergammaglobulinemia[248, 2]

Chanutin and his associates[53] have applied the ultracentrifuge to this

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2 [2]Gutman A B and Gutman E B Relation of Serum Calcium to Serum Albumin and Globulins *J Clin Investigation* 16 903 (1937)

[249]Greenberg D M The Interaction between the Alkali Earth Cations Particularly Calcium and Proteins *Advances in Protein Chem* 1 121 (1944)

[50]Drinker N Green A A and Hastings A B Equilibria between Calcium and Purified Globulins *J Biol Chem* 131 641 (1939)

[51]Svensson H Fractionation of Serum with Ammonium Sulfate and Water Dialysis Studied by Electrophoresis *J Biol Chem* 139 805 (1941)

[25] Rawson A J and Sunderman F W Studies in Serum Electrolytes XV The Calcium Binding Property of the Serum Proteins (Multiple Myeloma Lymphogranuloma Venereum and Sarcoidosis) *J Clin Investigation* 27 82 (1948)

[253]Ludewig S Chanutin A and Masket A V Studies on the Calcium Protein Relationship with the Aid of the Ultracentrifuge II Observations on Serum *J Biol Chem* 143 753 (1942)

problem but the resolving power of the ultracentrifuge in respect to the separation of the plasma proteins is limited Moreover these investigators did not make studies over a sufficient range of variation in plasma proteins to permit relevant conclusions

It would seem that this whole problem of the calcium protein complexes in plasma should be explored further particularly now that relatively homogeneous plasma protein fractions are available However difficulties due to the rapid decrease in calcium binding properties as the result of denaturation especially in the case of the albumins and to the lack of precision of the available methods for estimating the concentration of calcium ions among other technical problems still make for uncertainties

Fremont Smith Is it possible that any calcium could be bound to some substance other than protein and might therefore be diffusible if not ionic?

Gutman There is some suggestion I believe that cephalin binds calcium I do not know whether that report has ever been checked

Neuman Based on its acidic properties cephalin should bind calcium

Handler The amount of cephalin is so small that it should be insignificant in this regard

Neuman That is one aspect and another is that I rather doubt whether it would pass through the ultrafilter It is probably protein bound

Butler Did it bother you that in most of the diseases with high total protein and high total calcium level it is the globulin fraction that is high not the albumin? In liver tumor multiple myeloma kala azar and so forth the albumin is low the globulin is high and the calcium is high

Gutman I shall discuss this point in a moment Dr Butler

The Calcium Protein Relationships in Multiple Myeloma

In multiple myeloma associated with hyperglobulinemia (or Bence Jones proteinemia) and hypoalbuminemia there often is a coincidental rise in the serum calcium level However if one plots the total serum calcium concentration against the total serum protein concentration in such cases of multiple myeloma with both hyperproteinemia and hypercalcemia no linear or other relationship can be discerned (see *Figure 4* of Gutman and Gutman[4] which shows the distribution of 75 points representing the available data from the literature) complete scatter is obtained And of course there are many cases of multiple myeloma in which hypercalcemia is associated with normal serum protein level and in which hyperproteinemia is associated with normal serum calcium level

Howard That holds for sarcoidosis too I think

Gutman Under these circumstances it seems to me likely that the hypercalcemia of multiple myeloma reflects the mobilization of calcium by myeloma cells invading the skeleton similar to the hypercalcemia seen in association with extensive rapidly progressive osteolytic metastases (usually not accompanied by hyperproteinemia) and does not represent a pulling out of calcium from the bones by excessive concentrations of plasma proteins Ordinarily marked hyperproteinemia is due to hyper gammaglobulinemia and as I have pointed out the affinity of gamma globulins for calcium at the pH of the plasma appears to be very small

The Calcium Protein Relationships in Lymphogranuloma Venereum

Certainly in most diseases characterized by hyperglobulinemia hypercalcemia is not found unless there is as in multiple myeloma some involvement of bone by the underlying disease process We first became aware of this principle in the course of studies on the hyperproteinemia of lymphogranuloma venereum a disease which often is associated with marked hypergammaglobulinemia but which does not affect the skeleton In spite of the presence of serum protein levels as high as 11 2 gm per 100 cc with serum total globulin contents as high as 7 8 gm per 100 cc the serum calcium levels invariably remained within normal limits [2 254 255] Normal serum calcium levels are regularly observed also in association with the hyperglobulinemia of hepatic cirrhosis kala azar granuloma inguinale disseminated lupus erythematosus and other disorders Sarcoidosis possibly is an exception since as has already been pointed out little or no skeletal decalcification can be detected in many cases exhibiting hyper calcemia with or without marked hyperglobulinemia

Perhaps I can make this point more clearly by reference to Figure 75

Here the total serum protein level expressed in gm per 100 gm of serum water is plotted against the total serum calcium level expressed in mg per 100 gm of serum water The straight line represents the regression equation developed in the classic studies of Hastings Murray and Sendroy

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2 Williams P D and Gutman A B Hyperproteinemia with Reversal of the Albumin Globulin Ratio in Lymphogranuloma Inguinale *Proc Soc Exper Biol and Med* 34 91 (1936)

5 Gutman A B and Gutman E B, Calcium Protein Relation in Hyperproteinemia Total and Diffusible Serum Calcium in Lymphogranuloma Inguinale and Myeloma, *Proc Soc Exper Biol and Med* 35 511 (1936)

56 Hastings A B Murray C D and Sendroy J Jr Studies of the Solubility of Calcium Salts I The Solubility of Calcium Carbonate in Salt Solutions and Biological Fluids *J Biol Chem* 71 723 (1927)

$$\text{Total Ca} = m \bullet \text{total protein} + b$$

where *m* the slope of the line is a constant which defines the amount of calcium bound per unit of total protein and *b* the intercept on the ordinate is a constant which defines the diffusible (or ionized) calcium. It will be noted in Figure 75 that there is excellent agreement in respect to *the open circles* which represent the range of serum protein level given by nephrotic patients who have marked hypoproteinemia due to hypoalbuminemia and by normal subjects as the serum protein levels fall there is a concomitant decline in the serum calcium levels. *The solid dots* however which represent patients with lymph granuloma venereum and hyperproteinemia due to marked hypergammaglobulinemia together with substantially normal serum albumin levels do not fall on the line but rather uniformly below it there is no significant increase in the serum calcium level as the serum protein level rises.

The explanation for the discrepant behavior of the two sets of data in Figure 75 lies I believe in the different calcium binding properties of the plasma proteins in question. In the case of nephrosis the fall in the serum protein concentration is due to a decline in the serum *albumins* which appear to bind appreciable quantities of calcium hence the serum calcium level shows a correspondingly appreciable drop. In the case of lymphogranuloma venereum the increase in the serum protein concentration is not due to an increase in the albumins (I know of no disease characterized by hyperalbuminemia except for hemoconcentration) but to an increase in the *gamma globulins* which appear to bind very little calcium hence the serum calcium level shows no significant rise.

The Calcium Protein Relationships in Hepatic Cirrhosis

This difference is shown even more strikingly in Figure 76 in which the data on many cases of hepatic cirrhosis have been added to the plot. In advanced cirrhosis of the liver both marked hypergammaglobulinemia and definite hypoalbuminemia are apt to develop concurrently so that the total plasma protein concentration usually remains within normal limits or shows some elevation. The serum calcium concentration however is either low normal or (if the hypoalbuminemia is marked) somewhat depressed.

Figure 76 reveals that the points fall well below the line representing the expression based on a single factor *m* for the total serum protein.

Finally Figures 77 and 78 show the distribution of the points when the total serum calcium concentration in these cases is plotted against the total level of serum globulin and serum albumins respectively. The data indicate that very little of the calcium is bound to the gamma globulin in contrast to a significant amount of the calcium that is bound to the albumins.

While there is considerable scatter in both graphs it appears that the slope of a line through the points in Figure 77 would approximate zero indicating very

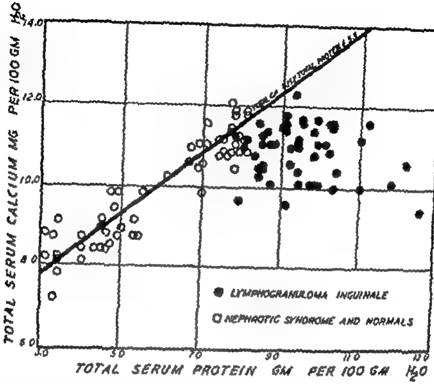

Fig 75 The Relationship of the Total Serum Calcium to the Total Serum Proteins in a Condition With Normal Proteinemia (Normal Subjects) in a Disease Presenting Hypoalbuminemia Without Hyperglobulinemia (The Nephrotic Syndrome) and in a Disease Presenting Hyperglobulinemia (Hypergammaglobulinemia) Without Significant Hypoalbuminemia (Lymphogranuloma Venereum)

The open circles represent the data from the normal subjects and *from the patients* with the nephrotic syndrome and *the solid circles* represent the data from the patients with lymphogranuloma venereum Note the linear distribution of the data *from the normal subjects and from the patients* with the nephrotic syndrome and the divergency from the linear distribution of the data from the patients with lymphogranuloma venereum

[Reproduced by permission from Gutman A B and Gutman E B Relation of Serum Calcium to Serum Albumins and Globulins *J Clin Investigation* 16 903 (1937)]

little of the calcium is bound to the gamma globulins whereas a line through the points in Figure 78 would show a distinct slope indicating a significant amount of the calcium is found to the albumins

Butler I am quite certain I can remember cases of liver disease with no metastases with high serum calcium values fitting in with the high

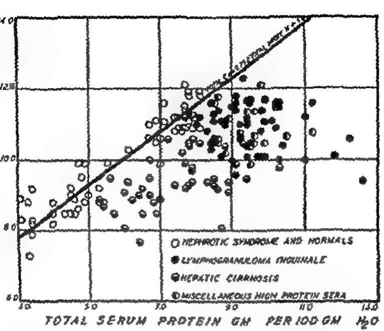

Fig 76 The Relationship of the Total Serum Calcium to the Total Serum Proteins in a Condition With Normal Proteinemia (Normal Subject), in a Disease Presenting Hypoalbuminemia Without Hyperglobulinemia (The Nephrotic Syndrome), in a Disease Presenting Hyperglobulinemia (Hypergammaglobulinemia) Without Significant Hypoalbuminemia (Lymphogranuloma Venereum) and in a Disease Presenting Marked Hyperglobulinemia (Hypergammaglobulinemia) With Distinct Hypoalbuminemia (Hepatic Cirrhosis)

The [illegible] *les* represent the data from the normal subjects and from the patients with the nephrotic syndrome, *the solid circles* present the data from the patients with lymphogranuloma venereum, and *the half-solid circles* represent the data from the patients with hepatic cirrhosis and from the patients with miscellaneous disorders [illegible] high protein [illegible]. Note that this is Fig. 5 with the addition of the data from the patients with hepatic cirrhosis and with miscellaneous disorders [illegible] high protein sera. Note the [illegible] low serum calcium level in hepatic [illegible].

[Reproduced by permission from Gutman, A. B. and Gutman, E. B.: Relation of Serum Calcium to Serum Albumin and Globulins. *J. Clin. Investigation* 16, 903 (1937).]

total protein and high total globulin level

Gutman: I am not familiar with that. May I make one other point however?

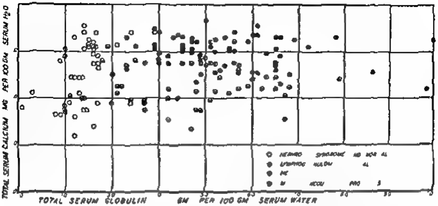

Fig 77 The Relationship of the Total Serum Calcium to the Total Serum Globulins in the Same Individuals Shown in Fig 76

Note the roughly linear dispersion of the data with a slope approximating zero

[Reproduced by permission from Gutman A B and Gutman E B Relation of *Serum Calcium* to Serum Albumin and Globulin *J Clin Investigation* 16 903 (1937)]

The Calcium Binding Capacity of Plasma Proteins

The amount of protein normally present in plasma is sufficient to enter into combination with much more calcium than is ordinarily present in the blood This is readily shown by the many experiments in which calcium salts were added to serum *in vitro* or *in vivo* and the amount of non filterable calcium was then found to be increased [7, 85] The same *thing happens in vivo when excessive calcium is mobilized from the bones* there is a redistribution of calcium with higher levels of both protein bound and ionized calcium in the serum

Conference Discussion

Armstrong When you inject serum albumin intravenously does the serum calcium level respond promptly by elevation?

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[7] Smith P G and ~ berger H R le and Non Diffusible Blood Serum Calcium Following Injecti n Salt *J Biol Chem* 96 245 (1932)

[5] Greenberg D on C L on of Calcium Proteinate and Colloidal Calcium the F cium in the Blood Stre ■ J *Physical Chem*

ns T d Ultrafiltration Studies on Cal Phosp m *ns Hosp* 91 1 (1952)

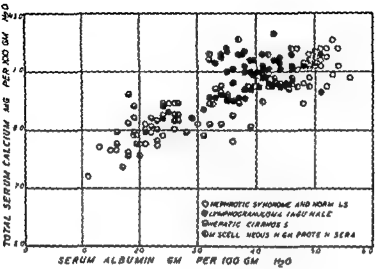

Fig 78 The Relationship of the Total Serum Calcium to the Serum Albumin in the Same Individual Shown in Fig 76

Note that in spite of much scatter a distinct trend with a steep slope is apparent

[Reproduced by permission from Gutman A B and Gutman E B Relation of Serum Calcium to Serum Albumins and Globulins *J Clin Invest* 16 903 (1937)]

Heinemann Yes it does

Gutman The elevation would not be more than a few hours would it

Heinemann It may persist for several weeks If 50 grams of serum albumin are administered intravenously daily there may be a rise in the serum calcium concentration to 12 mg per 100 cc or more The hypercalcemia is maintained so long as the elevated serum albumin levels are maintained with continued albumin infusion

Howard Have you ever produced hyperalbuminemia in that manner

Heinemann Yes

Howard And the serum then is hypercalcemic

Heinemann That is correct

Gutman Have you ever encountered this situation in disease states

Henneman The occasional concurrence of hyperalbuminemia and hyper calcemia in Burnett s syndrome [60] ■ another facet of this problem In this condition we believe that the hypercalcemia results from excessive calcium ingestion *plus* decreased urinary calcium excretion The cause of the hyperalbuminemia is not apparent

Gutman Perhaps it is associated with some degree of dehydration

Henneman Yes it might be

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[60] Burnett C H Commons R R Albright F and Howard J E Hypercalcemia without Hypercalcuria or Hypophosphatemia Calcinosis and Renal Insufficiency *New England J Med* 240 787 (1949)

SOME BASIC CONCEPTS CONCERNING CALCIUM

FRANKLIN C. McLEAN

From the Department of Physiology, University of Chicago School of Medicine, Chicago, Illinois

Armstrong: Dr. McLean, do you wish to make a comment at this point?

Base Bound to Protein

McLean: I would like to start first with some of the basic concepts. Dr. Gutman has referred to the fact that calcium is bound to various substances in the plasma, particularly a variety of proteins. I would like to examine the concept of "base bound to protein," since this term has led to considerable confusion in the past. It appears to have arisen as an abbreviated method of referring to the combining power of proteins for cations, and it actually expresses the number of negative valences free to neutralize electrically the positive valences of cations. In the usual instance, such as that of sodium and protein, there is complete or nearly complete dissociation in such a solution as that represented by plasma, and reference to sodium as "bound to protein" is misleading. This term, so popular a generation ago, seems to be losing ground. I do not hear it any more in this Conference and I hope that it will disappear from the literature.

Calcium, on the other hand, forms undissociated complexes with many organic acids, including protein. The dissociation constants differ a great deal according to the acid. The one that has the highest dissociation constant for its calcium complex, as far as I know, is citric acid, but if one examines other organic acids, one finds also that calcium forms an undissociated fraction with many, perhaps all, of them. There is a considerable variety of organic substances in the blood, of which many are capable of forming undissociated complexes with calcium, but for most of them the dissociation constant is such that only a small fraction of the calcium present can be held in undissociated form, with the result that not more than 1 to 2 (or at the most 5 to 10) per cent of the total calcium of the plasma can be accounted for in this way.

Ionization

Moreover, there has been an interesting change in the views of the

inorganic chemist with respect to ionization I am certain that everyone here was taught that all salts are strong electrolytes in the sense that it was believed that they were all completely dissociated in solution That idea is losing ground to the extent that it is now generally recognized that at least salts of divalent ions and particularly where two divalent ions positively and negatively charged are present in solution together are not completely dissociated Such a salt as calcium sulfate for example is now known not to be completely ionized in solution Instead there is some undissociated calcium sulfate and the salt although a relatively strong electrolyte has a dissociation constant just as does a weak electrolyte We were talking earlier in this conference about a complex of calcium and carbonate or bicarbonate In this respect we might better refer to this complex as an undissociated fraction of calcium carbonate when these two ions are present

But such fractions as this although of much interest are hardly of quantitative significance under physiological conditions It still remains true that there are only two important contributors to the formation of undissociated calcium combinations in the plasma These are protein and citrate and citrate is important only under certain special circumstances The rabbit and perhaps in addition some other herbivorous animals has enough citrate in its blood to result in a fairly large fraction of an undissociated calcium citrate complex

Nomogram for Calcium Protein Relationships in Serum

Let me substitute for Dr Gutman s curve (Figure 75) a nomogram (Figure 79) similar to one published by McLean and Hastings [61] which actually represents a family of curves of which Dr Gutman s is a special case

Each of these curves represents the equation $y = mx + b$ as indicated by Dr Gutman but with different values for the constants m and b It will be recalled that the constant b represents the Ca^{++} concentration and Dr Gutman s curve is for the case in which the Ca^{++} concentration is kept relatively constant by physiological regulation In order for one of these curves to represent the conditions in the plasma not only must the Ca^{++} concentration be kept relatively constant but the dissociation of calcium proteinate must be characterized at least statistically by the single constant m which determines the slope of the curve It will be recog

[61] McLean F C and Hastings A B The State of Calcium in Fluids of the Body I The Conditions Affecting the Ionization of Calcium J Biol Chem 108 285 (1935)

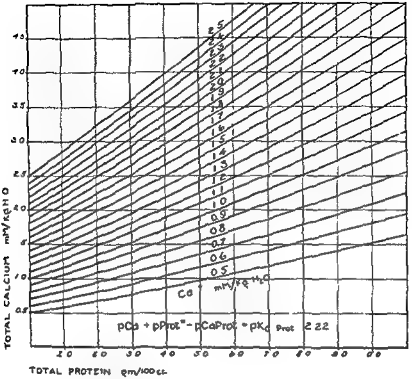

Fig 79 Cartesian Nomogram Illustrating Calcium Protein Relationships at Varying Calcium Ion Concentrations

mized therefore that the mass law equation as applied to calcium and protein in plasma is only an approximation and that it holds only in so far as the dissociation constant and the Ca^{++} concentration approach constancy. It certainly falls far short of describing the conditions in multiple myeloma, for example, when the dissociation constant as derived from the proteins of normal plasma no longer gives a correct characterization of the calcium protein relationships.

Conference Discussion

Neuman I want to make certain before the conference is over that we officially recognize for the record the importance of the work just discussed—the evidence of calcium-protein interaction. I think very few of us realize the significance of the time when that concept was formu-

inorganic chemist with respect to ionization I am certain that everyone here was taught that all salts are *strong electrolytes in the sense that it* was believed that they were all completely dissociated in solution That idea is losing ground to the extent that it is now generally recognized that at least salts of divalent ions and particularly where two divalent ions positively and negatively charged are present in solution together are not completely dissociated Such a salt as calcium sulfate for example is now known not to be completely ionized in solution Instead there is some undissociated calcium sulfate and the salt although a *relatively* strong electrolyte has a dissociation constant just as does a weak electrolyte We were talking earlier in this conference about a complex of calcium and carbonate or bicarbonate In this respect we might better refer to this complex as *an undissociated fraction of calcium carbonate* when the e two ions are present

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[201]McLean F C and Hastings A B The State of Calcium in Fluids of the Body I The Conditions Affecting the Ionization of Calcium *J Biol Chem* 108 285 (1935)

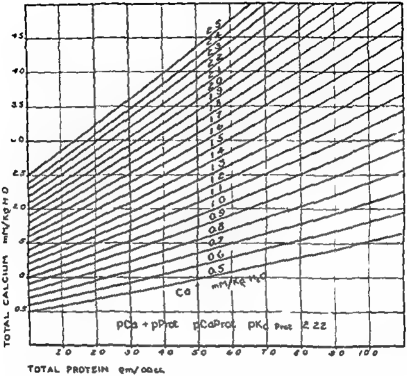

Fig 79 Cartesian Nomogram Illustrating Calcium Protein Relationship at Varying Calcium Ion Concentrations

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Conference Discussion

Neuman I want to make certain before the conference is over that we officially recognize for the record the importance of the work just discussed—the evidence of calcium protein interaction I think very few of us realize the significance of the time when that concept was formu

lated This work anteceded by a number of years the physicochemical knowledge and techniques we now possess At that time when that concept was advanced it was really unique Furthermore it has stood the test of time

The other comment I wish to make is that indeed the concept of dissociation is now unclear and the word complex is used loosely We have sodium chloride on the one extreme and I think calcium Versenate is very close to the other extreme Between them we have no terminology that indicates the degree of dissociation Anything that is relatively undissociated is called a complex

McLean Dr Gutman in his discussion said something about the capacity of protein to carry calcium in combination and stated that this property is not fully utilized This of course is true and it can be demonstrated very easily The McLean Hastings mass law equation is

$$\frac{[Ca^{++}] \times [Prot^{=}]}{[CaProt]} = K$$

in which K is the ionization constant of the weak electrolyte *CaProt* For our present purposes what this means is that as the Ca^{++} concentration changes the amount of calcium bound to any given amount of protein changes proportionately Or to state it in another way the amount of calcium that any amount of protein can carry in undissociated form is directly proportional to the Ca^{++} concentration If the Ca^{++} concentration were in a position to rise indefinitely which is not the case in the living organism then the amount of calcium bound to protein could also increase to many times the amount actually found in the blood at physiological Ca^{++} levels For the sake of clarity let me emphasize again that when I am speaking of calcium bound to protein I am referring to the undissociated calcium protein complex This complex dissociates instantaneously as calcium ions are removed from plasma or serum consequently the addition of oxalate to the serum leads to precipitation of all the calcium present including that previously present in the undissociated form

SERUM ALBUMIN AND BONE MATRIX [1, 2, 3]

FULLER ALBRIGHT, FREDERIC C. BARTTER,[8]
ELEANOR F. DEMPSEY, ANNE P. FORBES,
PHILIP H. HENNEMAN[9] and EDWARD C. REIFENSTEIN, JR.[10]

From the Medical Service of the Massachusetts General Hospital and the Department of Medicine, Harvard Medical School, Boston, Massachusetts

Armstrong: We will hear now the report which Dr. Henneman and Dr. Albright bring to us on the effect of the intravenous administration of serum albumin.

Henneman: We have divided our presentation into three parts.

I. The Metabolic Fates of Intravenously Administered Albumin

The intravenous administration of human serum albumin to patients on

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[1] Work described in this paper was done under grants from the American Cancer Society, the Rockefeller Foundation, the Josiah Macy, Jr. Foundation and the National Institutes of Health.

[2] The salt poor concentrated human serum albumin used in these studies was through the kindness of Dr. Charles Janeway of Harvard Medical School and Dr. Sam T. Gibson of the American Red Cross.

[3] Some of the experiments reported herein have appeared in previous publications.[4-7]

[4] Albright, F., Forbes, A. P., Bartter, F. C., Reifenstein, E. C., Jr., Bryant, D., Cox, L. D., and Dempsey, E. F.: Studies on the Fate of Intravenously Administered Human Plasma Proteins in Idiopathic Hypoproteinemia and in Osteoporosis. *Symposia on Nutrition, Volume II, Plasma Proteins*, pp. 155-134. The Robert Gould Research Foundation, Inc. (1950).

[5] Albright, F., Forbes, A. P., and Reifenstein, E. C., Jr.: The Fate of Plasma Protein Administered Intravenously. *Trans. Assoc. Am. Physicians* 59: 221 (1946).

[6] Albright, F., Reifenstein, E. C., Jr., and Forbes, A. P.: Further Analysis of the Fate of Intravenous Plasma. *TRANS. MACY CONFERENCE ON METABOLIC ASPECTS OF CONVALESCENCE* [illegible]: 134-148 (194[illegible]).

[7] Albright, F., Forbes, A. P., and Bartter, F. C.: Further Studies on the Fate of Intravenously Administered Human Serum Albumin. *TRANS. MACY CONFERENCE ON METABOLIC ASPECTS OF CONVALESCENCE* 17: 242-246 (1948).

[8] Senior Surgeon, U.S.P.H.S. Present address: National Heart Institute, Bethesda, Md.

[9] Damon Runyon Memorial Fund Research Fellow, 1950-1953.

[10] At present: Director, Division of Biological and Therapeutic Research, The Schering Corporation, 2 Broad Street, Bloomfield, N. J.

a constant metabolic regimen is followed by a rise in the serum level of albumin, decreases in the urinary excretion of phosphorus, potassium and calcium, and a somewhat tardy rise in the urinary nitrogen excretion. In addition the serum calcium level rises, and salt and water are retained, with a gain in weight and a fall in the hemoglobin concentration. In most instances there is a fall in the serum alkaline phosphatase level.

As described previously, we identify in the foregoing metabolic alterations three possible fates of intravenously administered albumin: burned, converted, and unchanged.

1) A portion of the albumin is catabolized and its nitrogen excreted in urine as urea. This fate has been termed *burning* and is measured in these studies by the increase in the nitrogen excretion above the average nitrogen excretion of the control periods.

2) Another fate of the albumin injected is *conversion* to protoplasm. Since protoplasm is phosphorus rich whereas serum albumin is phosphorus free, this process withdraws phosphorus from the metabolic pool and hence decreases phosphorus excretion. The deviation of the phosphorus balance from the average of the control balance levels is corrected for changes in the phosphorus balance which can be accounted for by the alterations in the calcium balance. This corrected phosphorus balance is then expressed as its protoplasmic equivalent in grams of nitrogen by multiplying by 14 (the ratio of phosphorus to nitrogen in protoplasm).

3) If one adds the nitrogen of albumin burned to the nitrogen equivalent of the phosphorus of conversion and then subtracts this sum from the total nitrogen injected, the remainder represents the nitrogen of *unchanged* albumin; this constitutes the third fate of intravenous albumin.

Thus

$$\text{Total N Injected} = \underset{\textit{BURNED}}{\left[\begin{matrix}\text{N} \\ \textit{excr.} \\ \textit{(over control)}\end{matrix}\right]} + 47\underset{\textit{CONVERTED}}{\left[\begin{matrix}\text{P} \\ \textit{decrease} \\ \textit{(from control)}\end{matrix} - \frac{\textit{(Ca balance)}}{2.33}\right]} + \underset{\textit{UNCHANGED}}{\left[\begin{matrix}\text{N} \\ \textit{(remainder)}\end{matrix}\right]}$$

In Figure 80 these changes are represented schematically as though they occurred sequentially rather than concurrently. The block at the top represents the nitrogen of the albumin administered intravenously. *The vertical bars* represent hypothetical balance data as they might occur for phosphorus and nitrogen. Next *the phosphorus and nitrogen balances are plotted as deviations from the average balance of the control period* rather than as absolute values. *The heavily lined rectangle* indicates the total nitrogen administered and to be accounted for. *The nitrogen equivalent of the phosphorus retained during albumin administration* is plotted downwards from above as albumin converted, while *the nitrogen of burning* is plotted upwards from below. Between these two areas is *the remainder (the unhatched area) of the nitrogen* which represents unchanged albumin.

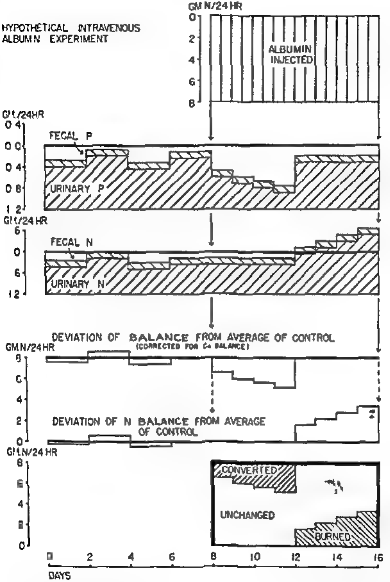


Fig 80 Hypothetical Experiment Illustrating the Derivation from the Nitrogen and the Phosphorus Balance Data of the Three Fates of Intravenously Administered Albumin

Note that the deviations from the average of the control balance rather than the balances as actually measured have been used as a basis for deriving the fate data For further discussion see text

II A Comparison of Oral and Intravenous Albumin Administration

Figure 81 presents data from parallel studies on a 31 year old female patient with idiopathic osteoporosis treated with identical quantities of albumin first intravenously and then orally. Figures 82 and 83 present interpretations derived from these data. With oral administration (see Figure 82) all of the albumin right from the first day was accounted for by burning *plus* conversion; with intravenous administration (see Figure 83) a large fraction remained unchanged. It is this unchanged fraction which undoubtedly accounts for the rise in the serum protein level with intravenous albumin (see Figure 85). It will be noted further that conversion was of the same order of magnitude in the two studies.

Prior to discussing the calcium balance data in these two experiments let us consider the original experiment of this series which emphasized the relation between the serum protein level and the calcium excretion.

III The Relationship Between the Serum Protein Level and the Calcium Balance

A EFFECTS OF PREGNANCY, INTRAVENOUS PLASMA AND NITROGEN INTAKE IN A PATIENT WITH IDIOPATHIC OSTEOPOROSIS

E. I., a 28 year old housewife (M.G.H. #399508) presented herself in 194? because of pain in the back and feet. In 1938 she jumped from a fence and thereafter noted a dull aching in her feet on prolonged standing. During her first pregnancy in 1939 she noted backache for the first time. In 1941 she fractured her left hip in a fall down three steps. At the time of admission she presented chemical findings characteristic of osteoporosis in that her serum calcium, phosphorus and alkaline phosphatase levels were normal. (By osteoporosis we mean deficient bone mass due to a failure of bone matrix formation as opposed to deficient bone mass due to an excessive rate of bone destruction or to a deficient calcification.) X rays demonstrated extensive osteoporosis with collapse of multiple vertebrae and a healed fracture of the left femur. In 1944 she again became pregnant and a low total serum protein level with a normal or elevated globulin fraction was noted.

Figure 84 illustrates the metabolic data obtained on patient L. L. while she was pregnant in 1944 and following a therapeutic abortion. Before the abortion she was in nitrogen equilibrium but negative calcium balance and had a low serum protein level. Following the termination of the pregnancy the serum protein level rose and the urinary calcium excretion fell. The administration of plasma further elevated the serum protein value and produced an additional slight decrease in the calcium excretion so that equilibrium was achieved. A low protein diet led to a fall in the serum protein level and a return to a n[illegible]ve calciu[illegible] subsequently a high protein die[illegible] did the op[illegible] striki[illegible]ship between the serum protein level and th[illegible]lance [illegible]e additional studies to be presented.

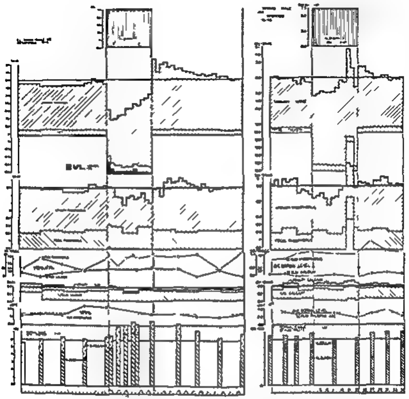
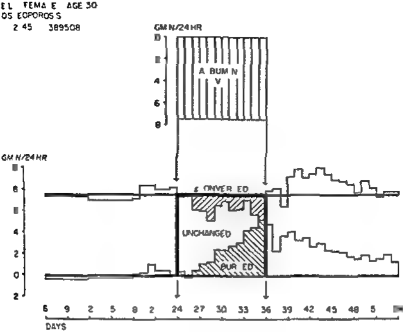


Fig 81 Parallel Experiments with Intravenous and Oral Albumin Administration on E L a Female Patient with Idiopathic Osteoporosis Metabolic Data for Nitrogen Phosphorus Calcium and Serum Proteins

Note the failure of the serum albumin level to increase with the oral albumin administration. The calcium data for these experiments are given in Fig 85. The decreased intake on days 1 and 2? of the oral albumin experiment was due to a gastric upset

12 DAYS OF ALBUMIN ADMINISTRATION IN A FEMALE PATIENT WITH IDIOPATHIC OSTEOPOROSIS

Figure 85 presents the calcium excretion during the 12 days of intravenous albumin administration in this same patient E L. Note the ~~profound fall in~~ the calcium excretion associated with a rising serum albumin level. The constancy of this relationship *plus* the fact that essentially all of the body calcium occurs in bone and teeth suggest that intravenously ad



F g 83 Fate Chart for the Intra e o Allu n l xper t Slo v n F g 81

N e ha he e a a cons le able an nt o a u use n l Comp tl la t w F g 8

n m stered a l u st n late lo for t

C 30 DA S OF ALBUMIN ADM NI TRAT ON N A FE AL PAT ENT WIT IDIOPATI C OSTEOPO OS S

The ext exper ent o tl s sane pat e L F wa f pe al er t le ause the pat e t was g ven the all tr e l for 30 lav l r the n d l e 10 da s of wl ch sl e re e ed a s all a l ratl er pote t d age of cort sone acetate orally In F gure 86 are pre ted tl e tl r e fate of t e ntravenou ly adn n tered ill un n n th s exper me t In F re 87 note the n al nega ve calc um l lan tl e n arke l fall tl e ur ary ca c m excre o rea l n zero tl e 5tl da an l tl e atta nn e t of a po t e calc un l ala ce wh ch pers ed for at le t 15 da s after tl e al l um n was d scont nued Note al the n a ked r n the le el of the serum p o e n espec a ly the a b n

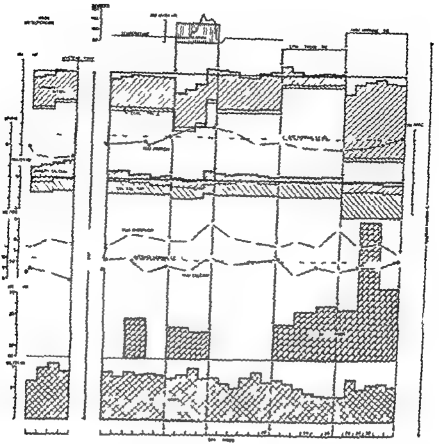


Fig 84 Metabolic Balance Data on E L a Female Patient with Idiopathic Osteoporosis During Pregnancy After a Therapeutic Abortion During the Intravenous Administration of Plasma and During Two Levels of Nitrogen Intake

This is *Experiment III A* in the text Note the low level of the serum protein during pregnancy the rise in the serum protein concentration after the termination of the pregnancy and the improvement in the calcium balance with the rising serum protein level

D 30 DAYS OF ALBUMIN ADMINISTRATION IN A FEMALE PATIENT WITH OSTEOGENESIS IMPERFECTA

A 24 year old woman E F (M G H #723271) with a congenital defect in bone formation known as osteogenesis imperfecta was given intravenously 8 8 grams of albumin nitrogen daily for 30 days Calculation of the

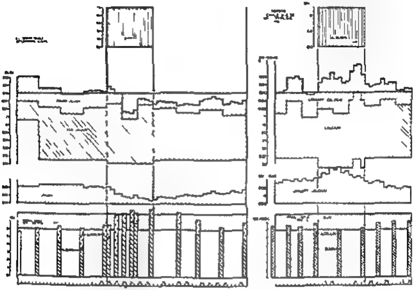


Fig 85 Metabolic Balance Data for Calcium and Serum Proteins on E L a Female Patient with Idiopathic Osteoporosis During the Intravenous and the Oral Administration of Albumin

See Fig 81 This is *Experiment III B* in the text For further discussion see text

[Reproduced by permission from Albright F Forbes A P Reifenstein E C Jr Bryant D Cox L D and Dempsey E F Studies on the Fate of Intravenously Administered Human Plasma Proteins in Idiopathic Hypoproteinemia and in Osteoporosis *Symposia on Nutrition* Vol II Plasma Proteins pp 155 194 Robert Gould Research Foundation Inc Charles C Thomas Publisher Springfield Illinois (1950)]

three fates of the intravenous albumin is shown in Figure 88 In Figure 89 are given the data pertaining to the calcium metabolism Note that the urinary calcium excretion again fell in a step-like manner until it reached zero on the 16th day of the albumin administration Note that in the control period following the administration there was a similar gradual increase in the urinary calcium excretion

E 12 DAYS OF ALBUMIN ADMINISTRATION IN A MALE PATIENT WITH IDIOPATHIC OSTEOPOROSIS

Figures 90 and 91 present a similar study in I I (M G H #601118) a 41 year old male patient with idiopathic osteoporosis The fate chart

is very similar to those of the two preceding studies The findings are self explanatory and support the previous observations

F 12 DAYS OF ALBUMIN ADMINISTRATION IN A PATIENT WITH POST MENOPAUSAL OSTEOPOROSIS COMPLICATED BY PAGET'S DISEASE

Figures 92 93 and 94 illustrate the metabolic effects of 12 days of intravenous albumin administration to S B a 60 year old patient (M C H #430664) with post menopausal osteoporosis and Paget's Disease Of particular interest is the marked retention of phosphorus and calcium superimposed upon an already lowered phosphorus and calcium excretion brought about by stilbestrol therapy Note the fall in the serum alkaline phosphatase level from 60 to 31 Bodansky Units during the albumin administration and the return of the level to higher values after the albumin was stopped The "rate" chart from this study (Figure 94) illustrates again the marked similarity of response in these patients with osteoporosis

G GLOBIN VERSUS ALBUMIN ADMINISTRATION IN A FEMALE PATIENT WITH IDIOPATHIC OSTEOPOROSIS

In Figures 95 and 96 are presented the data obtained in another experiment on E L the female patient with idiopathic osteoporosis She received intravenously 5 8 grams of nitrogen as Globin [7] (derived from human hemoglobin) for six days and somewhat later 8 3 grams of albumin nitrogen daily for 12 days The fall in the urinary calcium excretion obtained with the Globin was of the order of magnitude of that obtained with the albumin This raises some doubt as to the specificity of albumin as a precursor of bone matrix

Discussion

A QUANTITATIVE SIGNIFICANCE OF CALCIUM RETENTION

The patient in *Experiment III D* retained during the 30 days of treatment *plus* the 18 days of after control 1078 mg of calcium in excess of the slightly positive balance of the fore control period We believe that this represents a significant (*vide infra*) stimulation of osteogenesis but first let us consider how much calcium retention can be explained by calcium storage in plasma and extracellular fluid

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[7] The Globin used in this experiment was supplied through the courtesy of Dr William Boger of Sharp and Dohme Inc

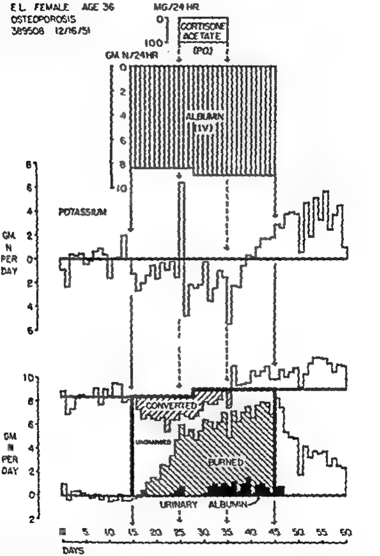

Fig 86 Fate Chart for the Intravenous Administration of Albumin for 30 Days on E. L., a Female Patient with Idiopathic Osteoporosis

This is *Experiment III C* in the text. Note that there was an immediate appearance of conversion which disappeared when burning reached a high level. Note that half of the injected albumin had been burned by the end of the injection period, that approximately 20 per cent was converted, and that a large portion remained unchanged. In general, the potassium balance followed the same pattern as the phosphorus balance.

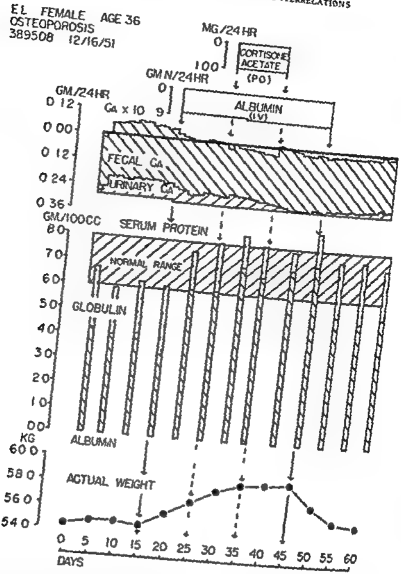

Fig 87 Metabolic Balance Data for Calcium and Serum Proteins for the Experiment Shown in Fig 86 on E L a Female Patient with Idiopathic Osteoporosis During the Intravenous Administration of Albumin for 30 Days

This is *Experiment III C* in the text. Note the fall in the urinary calcium excretion to zero the lack of change in the fecal calcium excretion and the tendency for the serum protein levels to plateau after 20 days of the albumin administration For further discussion see text

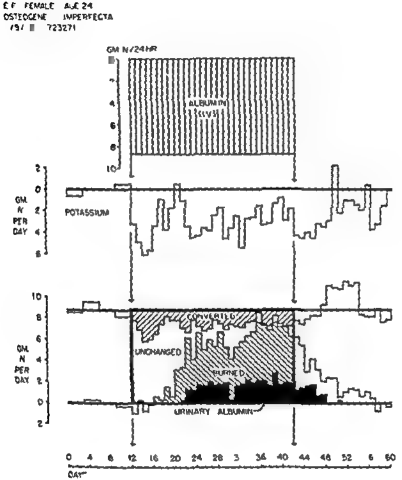

Fig 88 Iate Chart for the Intravenous Administration of Albumin for 30 Days on E F a Female Iatient with Osteogenesis Imperfecta

This is *Exper ent III D* in the text Note that the data are s milar to c mpa able data in *Expe iment III C* (Fig 86) Note further that conversi n continued to take place throughout the i ject on per and that burn g plus conversi n acco nted for virtually all of the injected album r by the 11th day of albumin admin istrat on The val d ty of the data is supported by the return of the n trogen and the phosphoru balances to the baseline during the control period following the adminis tration

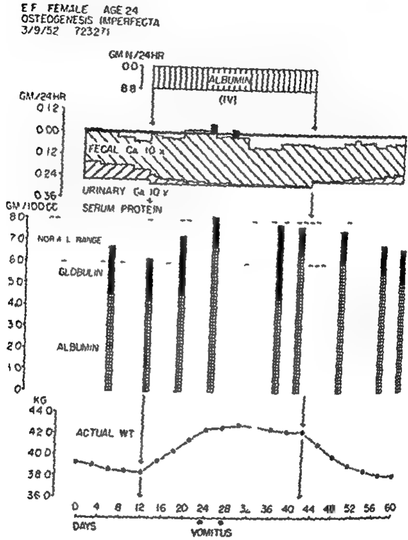

Fig 89 Metabolic Balance Data for Calcium and Serum Proteins for the Experiment Shown in Fig 86 on E F a Female Patient with Osteogenesis Imperfecta During the Intravenous Administration of Albumin for 30 Days

This is *Experiment III D* in the text Note the similarity of the data to those in Fig 87

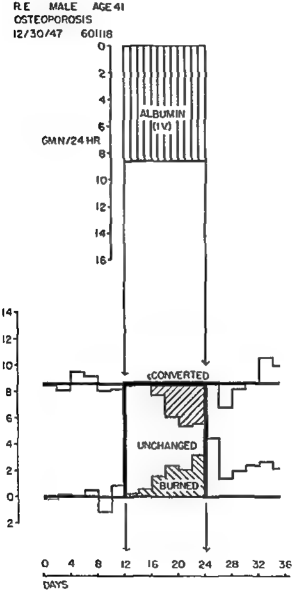

Fig 90 Fate Chart for the Intravenous Administration of Albumin for 12 Days on R. E., a Male Patient with Idiopathic Osteoporosis

This is *Experiment III F* in the text

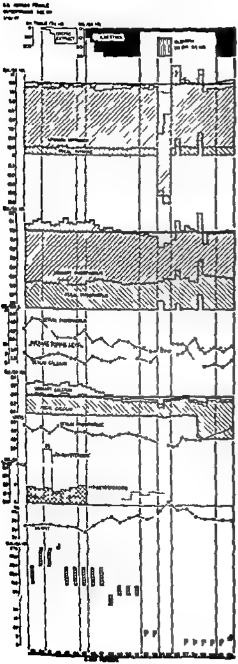

Fig 97 Metabolic Balance and Other Data on S B, a Female Patient with Post-Menopausal Osteoporosis Complicated by Paget's Disease, including Data Obtained During the Intravenous Administration of Albumin for 12 Days

This is *Experiment III F* in the text. The chart includes *(from the top to the bottom)* the data for the nitrogen balance, the phosphorus balance, the serum phosphorus level, the serum calcium level, the calcium balance, the serum alkaline phosphatase level, the urinary 11-oxysteroid excretion, the urinary 17-ketosteroid excretion and the urinary follicle stimulating hormone excretion. The effects are shown of the administration of an orchic extract, of stilbestrol at various dosage levels, and of serum albumin intravenously on these data.

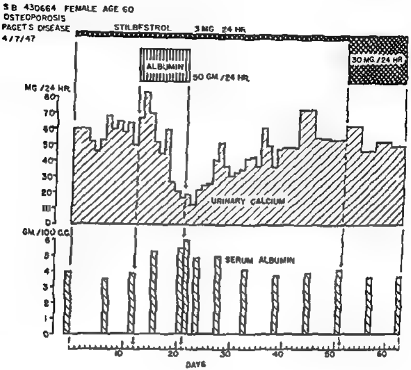

Fig 93 The Urinary Calcium Excretion and the Serum Albumin Levels in the Experiment Shown in Fig 92 on S I a Female Patient with Post Menopausal Osteoporosis Complicated by Paget s Disease During the Intravenous Administration of Albumin for 12 Days

This is *Experiment III F* in the text Note that 3 mg of stilbestrol by mouth daily had essentially the same effect on the calcium excretion as did 30 mg of stilbestrol by mouth daily and that the metabolic effect of the albumin was additive to that of the estrogen

[Reproduced by permission from Albright F Forbes A P Reifenstein E C Jr Bryant D Cox L D and Dempsey E F Studies on the Fate of Intravenously Administered Human Plasma Proteins in Idiopathic Hypoproteinemia and in Osteoporosis in *Symposia on Nutrition* Vol II Plasma Proteins pp 155 194 Robert Gould Research Foundation Inc Charles C Thomas Publisher Springfield Illinois (1950)]

The following calculations are an analysis of the possible mechanisms of calcium retention in *Experiment III D*

1 *1 Rise in Serum Calcium Concentration*

[2.7 mg / L. *(average rise in serum calcium concentration)*] × [5% × 40 Kg Body Wt *(average plasma or serum volume)*] = 54 mg

2 *Calcium Contained in Expanded Plasma Volume*

The observed fall in hemoglobin concentration from 13 to 9 grams per 100 ml would occur with an increase in plasma volume from a normal value of 2.0 L. to 3.5 L. Then the calculations are as follows

[1.5 L. *(probable increase in plasma volume)*] × [109 mg / L. *(maximum calcium concentration observed in serum)*] = 164 mg

3 *Rise in Extracellular Fluid Calcium Concentration*

[2.7 mg / L. *(assumed rise in extracellular fluid calcium concentration based on rise in serum calcium concentration)*] × [20% × 40 Kg Body Wt *(average extracellular fluid volume)*] = 16 mg

4 *Expansion of Extracellular Fluid Volume*

Sodium retained during albumin therapy in excess of control	=	659 mEq
Sodium in 1.5 L. increase in plasma volume (see *Calculation 2* above)	=	225 mEq
Difference (the amount of sodium contained in 2.9 L. of extracellular fluid)	=	433 mEq

[2.9 L. *(probable increase in extracellular fluid volume)*] × [76 mg / L. *(maximum extracellular fluid calcium concentration based on 70% of maximum serum calcium concentration)*] = 221 mg

5 *Total Calcium Accounted for by Storage in Plasma and Extracellular Fluid* = 655 mg

6 *Total Calcium Actually Retained* = 10.8 mg

---

[7] In *P* it is assumed that the fall in hemoglobin concentration was due entirely to dilution in an expanded plasma volume. In *C* it is assumed that all the increase in serum calcium was due to an increase in diffusible calcium and that extracellular fluid calcium would increase by a similar value. In *Exp. III G* are presented simultaneous determinations of diffusible and total serum calcium during albumin therapy. In *D* 70 per cent of the maximum concentration of serum calcium is used as the concentration of calcium in the expanded extracellular fluid in accordance with Howard's demonstration that as much as 70 per cent of serum calcium may be diffusible

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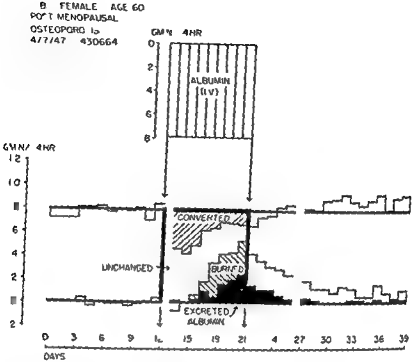

Fig 94 Fate Chart for the Intravenous Administration of Albumin for 12 Days on S B a Female Patient with Post Menopausal Osteoporosis Complicated by Paget s Disease

This is *Experiment III J* in the text The data for the first two days of albumin administration have been omitted because of incomplete intake and collections on these days

Similar calculations in the other experiments reported herein provide comparable data

It is a reasonable assumption that considerable calcium was retained as a consequence of increased osteogenesis Any form of binding of calcium by serum protein would not explain the step-like progressive fall in the urinary calcium excretion during the albumin administration or the sim

[274]Howard J E Studies on the Relationship of the Serum Calcium Level to Parathyroid Gland Function *TRANS MACY CONFERENCE ON METABOLIC INTERRELATIONS 4 140 1 3 (1952)*

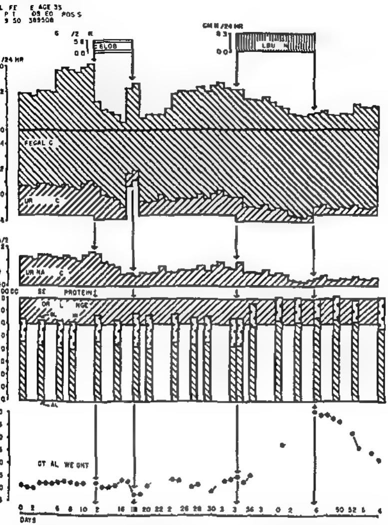

Fig 95 Metabolic Balance Data for Calcium Serum Proteins and Body Weight on L L a Female Patient with Idiopathic Osteoporosis During the Intravenous Administration of Glolin for 6 Days and of Albumin for 12 Days

This is *Experiment III G* in th text Th exper ment i on the same pat ent whose previous stud es a e hov n Fig III to 87 Note th marked lower g of the urinary calc um excreti n with Cl bin together with tl e fa lure of the serum protein levels to ri e and with the failure of the pat e t to ga n weight The decrease in ntake on day *17 and 18 was due to a reaction t the Globin*

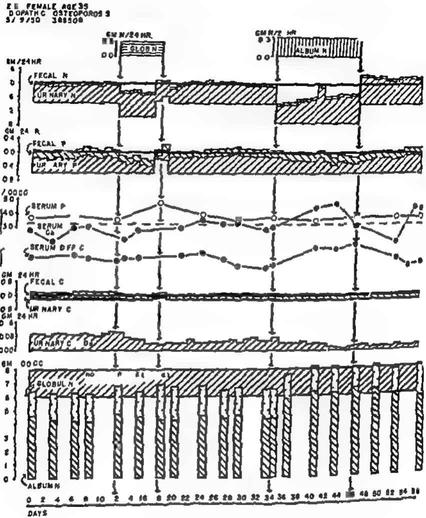

Fig 96 Metabolic Balance Data for Nitrogen Phosphorus and Calcium and Serum Levels of Calcium Phosphorus and Proteins for the Experiment Shown in Fig 95 on E. L. a Female Patient with Idiopathic Osteoporosis During the Intravenous Administration of Globin for 6 Days and of Albumin for 12 Days

This is *Experiment III G* in the text. The serum diffusible calcium concentration (*serum diff Ca*) was determined by ultrafiltration

ilar gradual rise on discontinuing the albumin. If albumin binding were the sole factor accounting for the decrease in the urinary calcium excretion one would expect a maximum depression of the urinary calcium excretion in the first day of albumin administration, less depression the longer the albumin were administered, and an increase above the control excretion (rebound) immediately on stopping the albumin administration. The shape of the calcium excretion curve during the albumin therapy and the failure to account for the retained calcium on the basis of storage in plasma or extracellular fluid strongly favor the view that intravenous albumin administration stimulates osteogenesis. Indeed the possibility is raised that albumin may be a transport form of bone matrix precursor.

B. DEPRESSION OF SERUM ALKALINE PHOSPHATASE

If increased osteogenesis occurred in these experiments one might expect an increase in the serum alkaline phosphatase level; the apparent fall appears paradoxical. The alkaline phosphatase data are collected in Figure 97.

C. SIMILARITY OF FATE OF ALBUMIN IN PATIENTS WITH OSTEOPOROSIS

Figure 98 summarizes the cumulative fates of albumin as percentages of total albumin administered by the fifth and tenth days of infusion in the group of patients with osteoporosis that has been studied. Note the similarity of pattern in all of the intravenous albumin experiments and the dissimilarity of the oral albumin and the globin experiments.

D. URINARY FECAL PARTITION OF CALCIUM

In these studies there was no demonstrable effect of the intravenous albumin on the fecal calcium excretion. From this it follows that the limit of effectiveness of albumin must be the height of the urinary calcium excretion (see Figures 97 and 89). In this respect serum albumin is less efficacious than estrogen, which decreases the fecal as well as the urinary calcium excretions.

Conclusions

Four patients with thin bones due to faulty matrix formation when treated with intravenous albumin demonstrated a marked calcium retention which is thought to reflect increased bone formation. The possibility is raised that serum albumin is a transport form of bone matrix precursor.

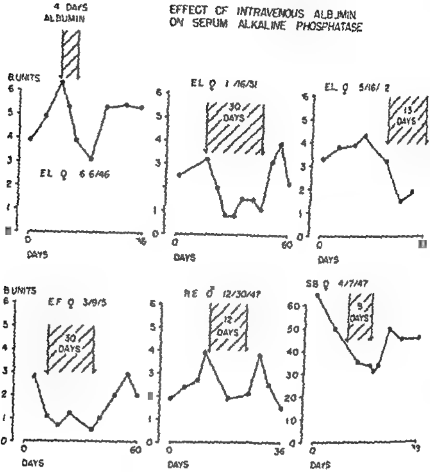

Fig 97 The Effect of Intravenously Administered Albumin on the Serum Alkaline Phosphatase Level

The upper three sets of data are on E L, a female patient with idiopathic osteoporosis, *the middle set* corresponds to the studies charted in Fig 8?, *the left lower set of data* is on E F, a female patient with osteogenesis imperfecta and correspond to the studies charted in Fig 89, *the middle lower set of data* is on R E, a male patient with idiopathic osteoporosis and corresponds to the studies charted in Fig ?, and *the right lower set of data* is on S B, a female with *post menopausal* osteoporosis and Paget's disease and correspond to the studies charted in Fig 9?. Note the change in the scale for the phosphatase on this last set of data on S B

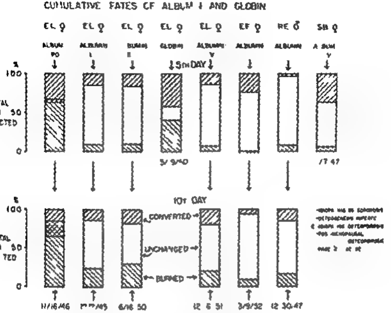

Fig 98 The Cumulative Fate Data of Orally and Intravenously Administered Albumin and of Intravenously Administered Globin on the Fifth Day and on the Tenth Day of Administration

Each column represents 100 per cent of the total nitrogen administered by the fifth or by the tenth day The columns correspond (*from left to right*) to the studies charted in Figs 82 85 86 88 90 and 94 respectively

Rebuttal

In evaluating the significance of the calcium retention in these experiments it must be recalled that the albumin was administered for relatively brief periods of time In the more lengthy experiments the depression of the urinary calcium excretion persisted as long as the albumin administration was continued we presume that this effect would continue for years if the albumin were continued this long the trabeculae acting as a storehouse for bone in bone The observed depression of the urinary calcium excretion reached the low values of 76 74 75 199 and 41 mg per day respectively in the five experiments reported If the albumin administration were continued such calcium retention would amount to 28 27 27 73

This section which was prepared after the Conference represents the result of the stimulation by the discussion which follows

and 15 grams of calcium, or 1.3 to 6.3% of the total body calcium yearly.[24a] Since the plasma volume, the extracellular fluid volume, and the serum calcium level did not continue to increase after the first 20 days of the albumin administration (when "burning" had increased to the extent of accounting for all the nitrogen administered daily), the calcium storage in these compartments would not be greater than that computed above and would represent an insignificant fraction of the calcium retained during very long albumin administration. When reduced to these terms, albumin compares favorably with estrogen in its ability to stimulate calcium retention and bone growth.

Conference Discussion

Armstrong: Over how long a period was the 1078 mg. of positive calcium balance obtained?

Henneman: Forty-five days.

Armstrong: This is equivalent to about a gram and a half of bone, isn't it? That is, the difference between the accounted for 700 mg. and the actually retained 1000 mg. would give enough calcium for about a gram and a half of bone?

Henneman: Yes.

Shorr: May I ask at this time whether these represent overall balances or just urinary calcium excretion changes? What about the total calcium balance?

Henneman: The step-like pattern is that of the urinary calcium excretion.

Shorr: And what was it again in total calcium balance as determined by the fecal and the urinary excretion?

Henneman: The 1078 mg. mentioned previously is the total positive calcium balance based on the calcium in the urine and the feces.

Handler: May I ask what is the error of your analytical determination of calcium?

Henneman: Two per cent.

Handler: I mean the percentage error in the determination. I do not recall whether you mentioned it, but assuming for the moment an intake of more or less one gram of calcium per day, that is, one gram per day for 45 days—

[24a] Shohl, Alfred T.: *Mineral Metabolism.* Reinhold Pub. Corp., New York (1939).

Henneman These patients were not on such large calcium intakes In most instances they received 200 to 350 mg per day

Handler Well that would be approximately 15 gram in 45 days Therefore this positive balance is 6 per cent of the total intake which is within the analytical error of any individual determination

Henneman I am not certain that I follow you

Handler I just wondered what the analytical error in your determination was and how real an event the indicated positive balance actually is

Armstrong Even assuming no analytical error if you subtract the 700 from 1000 approximately you have 300 mg of calcium that have been retained over and above that in the plasma or extracellular fluid If we take the large figure of 20 per cent for the calcium content of bone this retention then is equivalent to only a gram and a half of extra bone is it not Are my figures wrong?

Robinson No you are right There are about 6800 gm of bone in the body on the average

Sobel How much?

Neuman Nearly 7000 grams

Harrison Of bone? Oh yes

Neuman This is one and a half times this

Bartter Dr Handler you are assuming that all of the error is in the same direction You have all the daily values varying by six percent *plus*

Handler All that I was getting at was how significant is this number I am not challenging it I want to know the actual significance of this observation

Bartter You need a standard error of the two means to be able to estimate the reasonable error

Handler Agreed I was being suspicious that if we had such a computation we would find that we are discussing a very small phenomenon of little biological meaning

Howard The point that strikes me as most odd in these observations is the change in the renal attitude toward calcium after the administration of albumin You showed Dr Henneman that the total calcium in the serum rises and presumably therefore the diffusible fraction also rises but the calcium which appears in the urine falls That must mean then that the kidney has changed its method of handling calcium Is that right?

... further studies confirm ... serum calcium concentration ... that we must invoke a separate ... the decrease in the urinary

H...: There was no change in the ... reduction of the albumin? The urine ... alkalinity or the bicarbonate content did ... thing like that

H...: We have not followed the ... it would be altered by the albumin infusio

H...: You are stating that the diffi... that more is presented to the kidney ... but this unusual?

Henneman: Yes

K...: Dr Henneman what abou... accounted for by the nitrogen balance ... calcium balance?

Henneman: The positive phosphorus ... phorus retention which can be account... balance

H...: This is exactly the opposite ... nephrosis. I do not know what occurs ... with nephrosis but when you get a spont... the serum albumin level rises then the cal... relatively greater quantities after having ... That is not the opposite of Dr Henneman

H...: Does anyone know why th... ... in nephrosis?

H...: I seem to recall that Emerso... ... was disturbed. The stool calcium a... was ingested

...: Did this patient gain 8 kilogram

Henneman: The patient ... whose ... detail gained 4 kilograms during the albu

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[117] Emerson K Jr and Beckman W W ... scription of Abnormality in Calcium Metabol... *Investigation* 24 564 (1945)

the administration of 50 gm of salt poor albumin to patients with normal serum albumin levels results in a daily weight gain of 0 3 to 0 4 kilograms This is almost entirely accounted for by retention of salt and water

Butler You did not make any studies on what happened to glomerular filtration renal plasma flow and so forth?

Henneman No

Copp I should think this evidence indicates that the effect if any is on the kidney there is a decreased excretion with a higher serum level

Stevenson How high did the serum albumin go

Henneman Above 7 grams per 100 cc in some instances

Armstrong Were the patients edematous

Henneman None of our patients developed overt edema or symptoms of pulmonary congestion all of them had normal hearts and kidneys I believe Dr Bassett has observed some evidence of overloading of the circulation with intravenous albumin

Bassett Two of our patients developed edema and one developed a rather acute pulmonary edema one night As I recall it in regard to the kidney function there was an increase in the glomerular filtration rate in at least one of our patients after several injections of albumin presumably associated with the increase in plasma volume

Shorr How do you interpret the decrease in the alkaline phosphatase level? As a cessation of repair? I calculate that the amount of calcium absorbed in the bones was 7 mg a day Would that be enough to halt a reparative process in bone?

Henneman I do not know what the explanation is Does anybody know whether or not alkaline phosphatase is present in the extracellular fluid?

Armstrong Plasma is an extracellular fluid

Henneman Well does this enzyme readily pass from the extravascular fluid to the plasma?

Handler It is present in ascitic fluid This is as close as I can come

Henneman If alkaline phosphatase occurs in ascitic and other extravascular fluids dilution of the plasma by such fluids would not explain the fall in the serum alkaline phosphatase level

Howard We made one ultrafiltration experiment and found in it that alkaline phosphatase did not pass the ultrafilter

Freeman Does serum alkaline phosphatase readily pass through the [illegible]

Henneman The [illegible] patient had a serum alkaline phosphatase level [illegible] and we could not obtain any [illegible] the membrane. But this is only one observation.

Sobel To evaluate the serum alkaline phosphatase changes, one would first have to know what the effect of albumin [illegible] on the ability to measure the phosphatase chemically. Have you determined this by any chance

Henneman No, we have not done that, [illegible]

Sobel It is quite possible that the albumin affects the phosphatase determination *per se* and a correction would have to be made for the effect.

Henneman Do you know about that, Dr Gutman

Gutman I do not know of any effect of serum albumin on the alkaline phosphatase determination. In respect to the ultrafilterability of alkaline phosphatase it should be recalled that presumably it is, after all, a very large protein molecule like all enzymes. The few attempts I have made to ultrafilter alkaline phosphatase extract have indicated that the enzyme will not pass through a collodion membrane

Henneman Perhaps the phenomenon we observed is all dilution then

Shorr But how does the alkaline phosphatase get from the bone to the blood?

Henneman The same way that the [illegible] gets from the blood to the bone [Laughter]

Shorr Somehow or other it m[illegible] through the cap[illegible]

Preh[illegible]
[illegible]hib t[illegible] mh[illegible]
normal [illegible]
[illegible]her to l[illegible]
and sodium
[illegible] free

THE RELATIONSHIP OF VITAMIN D AND PARATHYROID HORMONE TO CITRATE METABOLISM, THE TREATMENT OF HUMAN RICKETS WITH CITRATE

HAROLD E HARRISON

From the Baltimore City Hospitals Baltimore Maryland

Armstrong Let us hear about another subject now I refer to the treatment of rickets with citrate—work which I think is fascinating Dr Harrison would you present your studies?

Harrison This work was stimulated by Dr Park who has asked us repeatedly to explain the mechanism of action of vitamin D Any approach that can be made to the understanding of the physiology of vitamin D may be of value in the understanding of the problem of the influence of activated sterols and steroids ... functions

One o ... de of action of vitamin D was the study of ... m ... may influence calcium distribu ... f undissociated calcium citrate ... years ago that the admin ... a rather unusual finding ... concomitant drop of the ... nued low level of the

... sm in Rickets

... would like to pre ... infants treated ... termination of ... average serum ... per 100 cc

... nc and

... ckets

Henneman Does serum alkaline phosphatase usually pass through the ultrafilter?

Howard The particular patient had a serum alkaline phosphatase level of 145 or 150 units and we could not obtain any in the ultrafiltrate But that is only one observation

Sobel To evaluate the serum alkaline phosphatase changes you would first have to know what the effect of albumin addition is on the ability to measure the phosphatase chemically Have you determined this by any chance?

Henneman No we have not done this as yet [19]

Sobel It is quite possible that the albumin affects the phosphatase determination *per se* and a correction would have to be made for this effect

Henneman Do you know about that Dr Gutman?

Gutman I do not know of any effect of serum albumin on the alkaline phosphatase determination In respect to the ultrafilterability of alkaline phosphatase it should be recalled that presum[illegible] ter all a very large protein molecule like all enzymes The [illegible] ave made to ultrafilter alkaline phosphatase extracts have [illegible] nzyme not pass through a collodion membrane

Henneman Perhaps the phenomen[illegible]

Shorr But how does he alkaline [illegible] blood?

Henneman The same way that th[illegible] bone [Laughter]

Shorr Somehow or other it mus[illegible]

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[19]Preliminary experiments indicate that t[illegible] inhibits alkaline phosphatase Thus inhibit[illegible] normal phosphatase activity and with sera either to liver disease or to bone disease Th[illegible] and sodium caprylate do not inhibit phosph[illegible] albumin free of these stabilizers does inhibit

THE RELATIONSHIP OF VITAMIN D AND 'ARATHYROID HORMONE TO CITRATE METABOLISM HE TREATMENT OF HUMAN RICKETS WITH CITRATE *

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One obvious approach to the mode of action of vitamin D was the study of its effect upon citrate metabolism Citrate may influence calcium distribution in the body by virtue of the formation of undissociated calcium citrate complexes Butler and Shohl showed many years ago that the administration of citrate to rachitic infants produced a rather unusual finding namely that healing of the rickets occurred with a concomitant drop of the serum calcium to hypocalcemic levels and with continued low levels of the serum phosphorus

The Effect of Vitamin D on the Citrate Metabolism in Rickets

Before considering the effect of citrate on rickets I should like to present some studies on the serum citrate levels of rachitic infants treated with vitamin D (Figure 00) The method used for the determination of citrate was that of Natelson Pincus and Lugovoy [2] The average serum citrate concentration in normal control infants is about 2 5 mg per 100 cc

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*Th s stud e we e supported by g ants from the Nutrition Foundation Inc and the Playtex Park Re earch Fou dati n

Sh hl A T and Butle A M C trates in the Treat ent of Infantile Rickets *New Engl d J Med* 220 515 (1939)

[2] Natelson S P cu J B and Lugovo J K M cr est mation of Cit c Ac d A New Colorimetric Reacti n fo P ntab omoacetone *J B ol Ch e* 175 745 (1948)

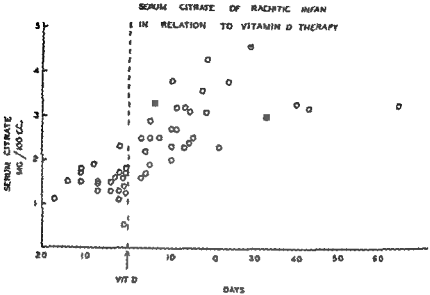

Fig 99 The Concentration of Citrate in the Serum of Ten Ra hitic Infants Before and After Treatment with Vitamin D

The vitamin D v as given as a ingle injection of 600 000 un ts

[Reproduced by permission from Harrison H E and Harri on H C Vita min D and Citrate Metaboli m *Yale J Biol and Med* 24 273 (195 1)]

In the group of 10 infants with vitamin D deficiency rickets the average concentration of serum citrate was low before treatment after vitamin D was administered the level rose in every subject and in some cases reache l supernormal levels The vitamin D was given intran uscularly as 600 000 units of vitamin D in oil In Figure 100 is shown an attempted correlation of the serum citrate and calcium concentrations Before treatment there was a wide variation in the concentration of the serum calcium from 5 mg per 100 cc to about 10 5 mg per 100 cc The serum citrate levels were low in all of these infants without any correlation with the serum calcium level Following treatment with vitamin D the levels of calcium and citrate ro e together so that there was a partial correlation between the serum citrate and calcium concentrations There does not seem however to be a simple relationship bet een the citrate and the calcium in the serum although procedures which increase the serum calcium level also tend to raise the serum citrate level

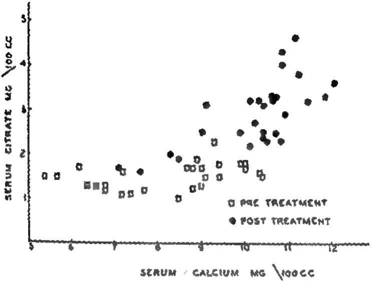

Fig 100 The Relationship of the Concentration of the Serum Citrate to That of the Serum Calcium in Rachitic Infants Before and After Treatment with Vitamin D

The open squares represent the values before treatment *the solid circles* represent the values after treatment The vitamin D was given as a single injection of 600 000 units

(Reproduced by permission from Harrison H E and Harrison H C Vitamin D and Citrate Metabolism *Yale J Biol and Med* 4 273 (19))

The Effect of Citrate Administration on Vitamin D Deficiency Rickets

In Figure 101 is summarized the effect of citrate in the treatment of an infant with vitamin D deficiency rickets Before treatment the concentration of calcium was about 9 mg per 100 cc and the serum phosphorus level was approximately 3 mg per 100 cc although the serum citrate concentration was 2 mg per 100 cc which is in the normal range

Citrate was given orally as a solution of citric acid and trisodium citrate (in equimolar amounts) in a dosage of 50 mM of citrate per day Following the feeding of citrate the serum calcium level dropped rapidly to a minimum value of 6 5 mg per 100 cc without any appreciable change in the concentrations of the serum phosphorus or citrate The concentration of the serum calcium then rose to between 8 and 9 mg per 100 cc and remained at this level while the serum phosphorus concentration varied

between 2 5 and 3 0 mg per 100 cc During this 4 week period x ray evidence of calcification of rachitic cartilage was observed In Figure 101 it can be seen that throughout this time the excretion of calcium in the urine remained extremely low the urinary excretion of phosphorus showed no consistent change while the excretion of citrate rose gradually

The points to be emphasized are 1) that the initial response of the rachitic infant to the feeding of a citric acid sodium citrate mixture was a drop in the serum calcium level and 2) that deposition of bone salts in rachitic cartilage as visualized by x ray occurred despite concentrations of calcium and phosphorus in the serum considerably below the levels usually considered necessary for calcification Both of these phenomena can be explained by the hypothesis that one effect of feeding the citric acid sodium citrate mixture is to make the rachitic cartilage more calcifiable so that calcium salts are rapidly removed from the body fluids

Howard Dr Harrison do the balances of calcium and of phosphorus change during the citrate administration even though the serum levels do not?

Harrison I cannot answer that question We were not able to do accurate balance studies in these infants

Howard Do you know Dr Butler

Butler We did not do balance studies either

Armstrong You are giving a considerable amount of other substances as well as citrate

Harrison Yes a considerable quantity of sodium

Armstrong I suppose you controlled this factor

Harrison Not in the infant There are studies in rats in which it has been shown that sodium alone does not cause healing of rickets Citric acid alone also causes little or no healing of rickets In contrast the mixture of the two causes a very decided healing of rickets For these infants however we have not had a control period on sodium alone That is an important point

Armstrong To control the study properly you would have to give the sodium with a metabolizable ion I should imagine such as the lactate

Harrison This has been done in rats Tartaric acid sodium tartrate mixtures also can produce healing of rickets but mixture of other organic acids with their sodium salts such as malonic malic and succinic acids have not caused healing of rickets in rats

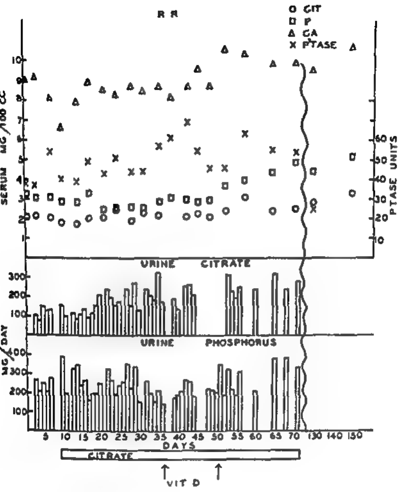

Fig 101 The Effect of Citrate Feeding in an Infant with Rickets

The columns represent the urinary excretion of phosphorus and of citrate *the open circles* represent the serum citrate level *the open squares* represent the serum phosphorus level *the crosses* represent the serum alkaline phosphatase level (in Bodansky units) *the open triangles* represent the serum calcium levels *the open block at the bottom of the figure* represents the period of feeding 50 mM per day of citrate as an equimolar mixture of citric acid and trisodium citrate and *each of the arrows at the bottom of the figure* represents an injection of 600 000 units of vitamin D

[Reproduced by permission from Harrison H E and Harrison, H C Further Studies of the Effects of Citrate Feeding on the Calcium Phosphorus and Citrate Metabolism of Rachitic Infants *J Ped* 41 756 (1952)]

Newman Do these mixtures improve intestinal absorption? Tartrate of course forms a complex with calcium

Harrison Presumably yes but no actual determinations have been made

Urist Our experience has been that the parenteral administration of citric acid will produce healing of rickets in rats but much less effectively than sodium citrate

Harrison Citric acid is much less effective In our own studies in rats citric acid alone has had little anti rachitic effect In the literature there is some disagreement In some studies citric acid without added sodium has produced healing of rickets in rats but has been less effective than mixtures of citrate and sodium or citrate and potassium The finding that interested us was the rapid drop in the serum calcium levels when the citrate was fed If citrate acts as a complexing agent in the intestine and thus improves the absorption of calcium how can one explain this decrease in the serum calcium concentration?

Sobel Did the drop take place after the healing or before it?

Harrison The fall took place before we could demonstrate healing by x ray

Sobel I asked the question because in following the course of healing by histochemical method one finds this sequence the calcium and phosphorus product keeps rising then just at the time when you can detect histological healing the product drops and after that it goes up again

Harrison In these infants the serum calcium concentration decreased as early as 24 hours after starting the citrate feeding It would not be possible to demonstrate healing as early as this

Follis I do not wish to set a definite number of days for the appearance of healing but it is quite possible that the x ray evidence is about two weeks late

Harrison Oh yes the x ray evidence is late But the changes I have mentioned occurred within 24 hours after the citrate administration was started You made the same observation Dr Butler

Urist In unpublished experiments on experimental animals we found a positive line test after four days of treatment with citrate ions introduced parenterally

Harrison Yes that is correct

Urist This treatment produced calcification *in vivo* comparable to a four plus line test

Shorr Dr Harrison did you measure the urinary calcium excretion

Harrison Yes the urinary calcium excretion was measured It was very low throughout this period and remained unchanged It was so low I did not put the values on the chart (Figure 101)

Butler Was there a drop in the serum calcium level when you gave the patients vitamin D?

Harrison We have not detected a decrease in the serum calcium values when we gave large doses of vitamin D Dr Stearns some years ago reported a marked drop in the serum calcium levels of rachitic children given small doses of vitamin D

Figure 102 illustrates in another rachitic infant given the citric acid sodium citrate mixture findings which are similar to those in the previous case Again there was an almost immediate drop in the values for the serum calcium level with no change in the serum phosphorus level Deposition of bone salts in the rachitic cartilage was seen by x ray during the period when the serum calcium level was about 8 mg per 100 cc and the serum phosphorus concentration was about 3 mg per 100 cc The level of the alkaline phosphatase activity which was extraordinarily high at the start (180 Bodansky units) decreased progressively during the period of citrate treatment The citrate feeding was continued for a period of 40 days without evidence of an elevation of the serum phosphorus levels However Dr Park has stated that if the citrate treatment is continued the serum phosphorus and calcium concentrations eventually do rise

These studies do not answer the question as to the mechanism of action of vitamin D We are not certain that the rise in the serum citrate levels following vitamin D therapy or the effect of the citrate on the healing of the rickets are evidences of a direct role of vitamin D in the intermediary metabolism of citrate It should be possible to determine how vitamin D does influence the cellular metabolic processes and whether or not the changes in the citrate level are manifestations of this effect We have hoped that the studies of Zetterstrom[2] which indicated that phosphorylated vitamin D could be shown *in vitro* to influence cell enzyme system might be the answer but we have not been able to obtain a sample of phosphorylated vitamin D which has the properties described by Zetterstrom

Folts Have you attempted to make this preparation of vitamin D?

Zetterstrom R Activation of Aerobic Oxidation in Kidney Mitochondria by Phosphorylated Vitamin D *Acta Chem Scand* 5 343 (1951)

Harrison Not ourselves An organic chemist associated with a commercial laboratory has been trying to prepare it without success

The Effect of Parathyroid Extract and of Vitamin D on the Citrate Metabolism in Hypoparathyroidism

I should like to report some studies on citrate metabolism in hypoparathyroidism Figure 103 illustrates the changes in the serum calcium phosphorus and citrate levels and in the urinary excretion of citrate in a child with hypoparathyroidism The serum citrate values initially were found to be below the normal levels When 10 cc of parathyroid extract were injected intramuscularly daily for 4 days a sharp increase in the urinary citrate excretion was observed and a rise in the serum citrate concentration from the initially low level to the normal range occurred along with the expected increase in the serum calcium concentration The urinary excretion of calcium did not change from the low levels of 10 to 15 mg per day When parathyroid extract was discontinued the serum calcium levels quickly returned to the pre treatment values the urinary excretion of citrate decreased but the concentration of serum citrate did not drop A prolonged period of Benemid treatment followed We were unable to confirm the observations of Hoffman[2] that in hypoparathyroid subjects Benemid increased the urinary excretion of phosphate and decreased the concentration of the serum phosphorus When vitamin D therapy was given in large doses an increase in the urinary citrate excretion again was found with a further rise in the serum citrate level and a rapid increase in the concentration of the serum calcium to the normal level

The Citrate Metabolism in Renal Tubular Acidosis with Rickets

Some studies on the citrate metabolism of an infant with renal tubular acidosis and rickets also may be of interest The infant had a persistent hyperchloremic acidosis with polyuria and a urine pH that always was close to 7 As shown in Figure 104 the serum calcium values were normal but the serum phosphorus concentrations and the serum citrate levels were low Two injections of vitamin D totalling 1 200 000 units had no effect upon either the serum phosphorus or citrate level When 40 cc of molar sodium lactate and 50 000 units of vitamin D were given daily the serum phosphorus concentrations rose to the normal range as did the serum citrate values The urinary citrate excretion was negligible before treatment and interestingly enough did not increase with the combined vita

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[2] Hoffman W S Fascale L and Dubin A Effect of Benemid on Phosphate Excretion in Parathyroid Tetany *Fed Proc* 11 231 (1952)

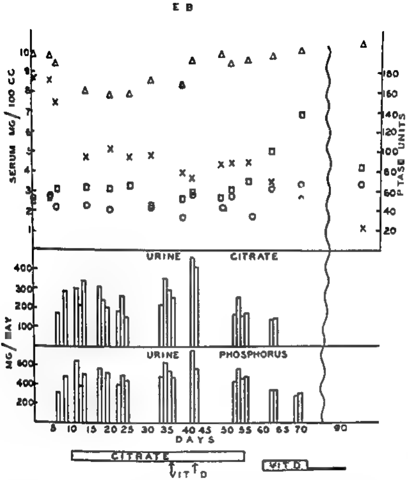

Fig 102 The Effect of Citrate Feeding in an Infant with Rickets

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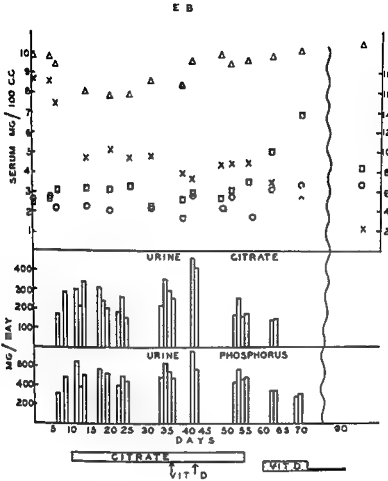
263 Hoffman W S Pascale L and Dubin A Effect of Benemid on Phosphate Excretion in Parathyroid Tetany F d Proc 11 231 (1952)



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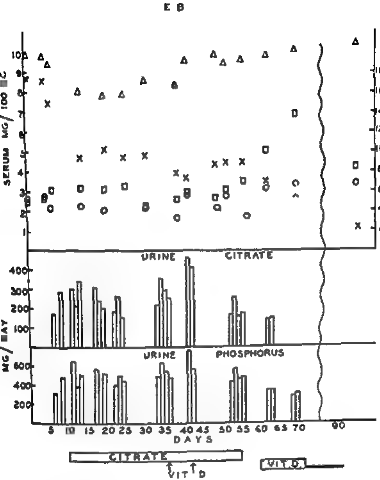

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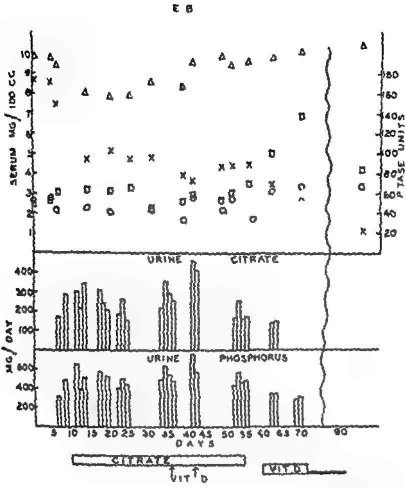

Fig 10 The Effect of Citrate Feeding in an Infant with Rickets

The columns represent the urinary excretion of phosphorus and of citrate *the open circles* represent the serum citrate level *the open squares* represent the serum phosphorus levels *the crosses* represent the serum alkaline phosphatase levels (in Bodansky Units) *the open triangles* represent the serum calcium levels *the open block at the bottom of the figure* represents the period of feeding 50 mM per day of citrate as an equimolar mixture of citric acid and trisodium citrate *each of the arrows at the bottom of the figure* represents an injection of 600 000 units of vitamin D and *the dotted block at the bottom of the figure* represents the period of the oral administration of Vitamin D at first 50 000 units per day and later 5 000 units per day

[Reproduced by permission from Harrison H E and Harrison H C Further Studies of the Effects of Citrate Feeding on [illegible] Phosphorus and Citrate Metabolism of Rachitic Infant *J Ped* 41 756

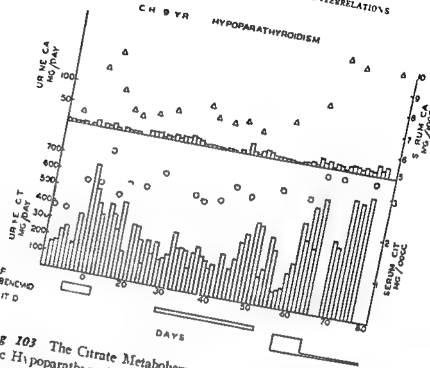

Fig 103 The Citrate Metabolism in a Nine Year Old Child with Idiopathic Hypoparathyroidism

The columns in the lower half of the figure represent the urinary excretion of citrate the open circles represent the serum citrate levels the columns in the upper half of the figure represent the urinary excretion of calcium the open triangles represent the serum calcium levels and *the open blocks at the bottom of the figure* represent periods of treatment *P E* — parathyroid extract (10 cc per day) *Benemid* (2 gm per day) and *Vit D* — vitamin D (800 000 units per day for 7 days and 100 000 units per day thereafter)

min D and sodium lactate therapy This child has never excreted any appreciable amount of citrate in the urine

Conference Discussion

Shorr An uninfected urine?

Harrison Yes collected with acid

Armstrong Was the urine acid?

Harrison The urine as voided has always been neutral or alkaline With sodium lactate therapy the urine was alkaline with a pH of about 8

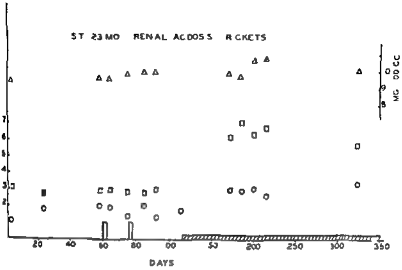

Fig 104 The Concentra on of C trate Pl o pl oru and Ca um n the Ser m of a 23 Month Old Cl ld w th Renal 1u lar Ac dos s and R ckets

T e op *l s* rep e ent the se um c a e le e *op n sq a s* ep e n he e um p o pho us le els *t e op t ang s* ep e ent he e um a um le e s *a h of he op n o a he bo ton of e fig e* ep e en s n ect on o 600 000 un s of v am D and *h ha l d o u n at he bo o n of figu* ep e en a p od of ea men w th mola sod um lac a e 40 c p day plu v am n D 50 000 un s per day)

B tler Suppose you had do e tl study v tho t tl e tam D a d w tl ju t so l um lactate *l at would l a e happened?*

Ha riso The ac do would ha e been corrected and o er a per od of t e the r ckets probal ly would ha e hea ed altho gh I am not cert n tl at tl e rach t c les o o ld l a e healed completely w thout extra v a m n D

Butle I o der why we so often g e huge do es ot tan D tl ese pa ents I lo not know that a o e l a pro e l that tl ey are neces ary

Sl orr I can co firm all of ur ob er at on on tl e relat onsh p be tween tl e l lood calc n and c tr e ac d le els Tl e an e n tl e ame l rect v l great regular ty the t c ac l le el fol ows an cl a ge n tl e cal u le el tl t are d ce l for exa ple l y the ren al of a paratl ro d tun or As you d ate Dr Harr o the relat onsl p

Harrison The urinary excretion of calcium was not unusually high

Ho ard These patients usually have a very large excretion of calcium

Harrison Yes they usually have a very high urinary calcium value In this patient the urinary calcium excretion was not very striking as I remember it but I do not have the figures here

STUDIES ON THE PURIFICATION OF PARATHYROID EXTRACT[284]

PHILIP HANDLER, DAVID V. COHN[285] and A. F. DRATZ

*From the Departments of Biochemistry and Nutrition
Duke University School of Medicine
Durham, North Carolina*

Armstrong: I think this would be a good point to hear from Dr. Philip Handler who I understand has some exciting results about the purification of parathyroid extract or at least the separation of it into more than one component.

Handler: Our study of these problems arose primarily from an interest in renal physiology and biochemistry rather than from the fact that the parathyroid hormone is related to the metabolism of bone. Some years ago we embarked on an investigation of the behavior of the kidney from various standpoints. We have been interested in renal hypertension for some time. We also have been concerned with the metabolism of the kidney *per se* and have studied the latter from several approaches.

The Problems in Renal Physiology Which Resulted in the Present Investigations

Perhaps naively we thought that we might be able to get at the nature of the mechanism whereby phosphate reabsorption occurs in the kidney using tracer studies with P^{32}. This was a rather ambitious project. We had hoped that we could do one experiment and solve several problems; in point of fact we obtained a great deal of data and succeeded in solving no problems whatsoever.

The problems were: a) Is the scheme of glycolysis which has been worked out by *in vitro* experimentation applicable to events occurring in any organ *in vivo*? b) Can the rate of turnover of adenosine triphosphate in the kidney be related to the actual thermodynamic work which the organ is being asked to perform at any given time? c) What is the mechanism of glucose reabsorption in the kidney? d) What is the mechanism of phosphate reabsorption in the kidney?

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[284] This work has been supported by the U. S. Atomic Energy Commission under contract AT (40-1) 289 with Duke University.

[285] Much of the work was performed during the tenure of Dr. Cohn as an Atomic Energy Commission Pre-Doctoral Fellow.

These studies were performed with Dr. A. F. Dratz. The chief difficulty proved to lie in the fact that events in the kidney occur with enormous rapidity. If we could slow them down, if by some means we could get the kidney to operate at perhaps 10° instead of 37°, we might solve some of these problems, but at 37° the processes in the kidney occur so rapidly that solution of these problems by available techniques seems impossible.

The Incorporation of Radiophosphorus (from Inorganic Orthophosphate) into Organic Phosphate Compounds in the Kidney

Essentially our procedure consisted of administering P^{32} as inorganic orthophosphate intravenously to a series of laparotomized dogs and of fractionating their kidneys for inorganic phosphate, adenosine triphosphate, glucose 1-phosphate, and glucose 6-phosphate, as well as a number of fractions irrelevant to this discussion. The specific activity of each fraction was then determined. Kidneys were taken for analysis at intervals from 5 to 120 minutes after P^{32} administration. These results have been published.[26]

An active reabsorptive process for phosphate in the kidney (and we all believe in its existence) implies the conversion of inorganic orthophosphate in the glomerular filtrate into something which is not inorganic orthophosphate as it goes through the cells of the tubular epithelium with a reconversion to its original form before it emerges on the other side. The only substance which we could find with a specific activity high enough to serve such a function was adenosine triphosphate. No other compound that we obtained fulfilled the necessary criteria. These criteria probably are known to most of you and were published by Zilversmit[27] some time ago. Unfortunately, adenosine triphosphate present in the kidney was demonstrated formerly to be not a single entity but a mixture of two or more forms of adenosine triphosphate existing in different pools which turn over at different rates. This may reflect merely the cellular heterogeneity of the kidney, or it may reflect the fact that within any cell you have independent pools, such as those in the mitochondria and outside the mitochondria, turning over at different rates.

With respect to the problem of glucose reabsorption, our efforts have been rewarded only by the finding that, if glucose 6-phosphate formation is obligatory to this purpose, only a minute fraction of the total glucose 6-

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[26] Dratz, A. F., and Handler, P.: Renal Phosphate and Carbohydrate Metabolism Studied with the Aid of Radiophosphorus. J. Biol. Chem. 197: 419 (1952).

[27] Zilversmit, D. B., Entenman, C., and Fishler, M. C.: On Calculation of "Turnover Time" and "Turnover Rate" from Experiments Involving Use of Labeling Agents. J. Gen. Physiol. 26: 325 (1943).

phosphate of the kidney is so engaged The bulk of this material in the kidney turns over at a rate entirely inadequate to meet the demands for glucose reabsorption We cannot of course rule out the possibility of a minute fraction turning over at an extremely rapid rate

The Effects of Parathyroid Extract on the Renal Excretory Mechanisms for Phosphate

At the same time we were engaged in another study in parallel with this[288] We thought that if we could establish how the kidney reabsorbs phosphate we might then observe what the effect of parathyroid hormone is on the active system thus engaged Initially we set out to demonstrate to our own satisfaction that there is something in parathyroid extract which does inhibit tubular reabsorption of phosphate At the time we started this work it was not clearly established in the literature that this process really does occur Perhaps our choice of the dog as the subject for these studies was unfortunate However it did lead Dr David V Cohn and me into several situations which proved to be amusing

The first event which occurred if a dog was given more than 100 units of parathyroid extract intravenously was an almost complete shutdown of glomerular filtration This lasted from 10 minutes to 30 minutes during this time the animal was anuric and there being no filtration there was no reabsorption either After that in most of our animals there occurred a rebound with glomerular filtration increased above the control baseline The increase was usually of the order of 20 per cent above the original value and persisted for a period of time that varied all the way from 30 minutes to 5 or 6 hours with no particular consistency not even within the same animal in different runs Under these conditions there was also a phosphaturia (Figure 105) However the phosphaturia generally was not the result of the impairment of tubular reabsorption but was caused by the increase in glomerular filtration thus we frequently obtained a phosphaturia in such animals in the presence of an absolute increase in tubular phosphate reabsorption[289]

Continuing such studies however we were able to demonstrate that if instead of giving the material intravenously we gave it intramuscularly in the same dosage then we obtained a perfectly satisfactory inhibition of

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[288]Our thanks are due to Armour and Co and Eli Lilly and Co for generous supplies of parathyroid glands and parathyroid extract respectively

[289]Handler P Cohn D V and DeMaria W J A Effect of Parathyroid Extract on Renal Excretion of Phosphate *Am J Physiol* 165 434 (1951)

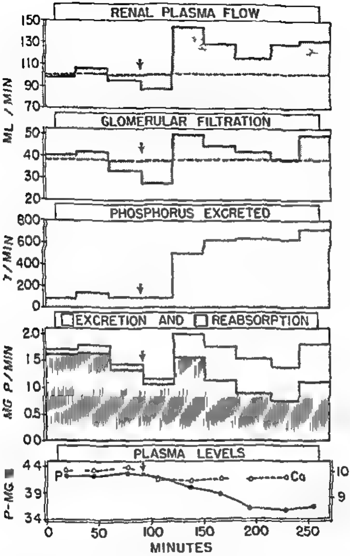

Fig 103 The Effect of Intravenously Administered Parathyroid Extract on the Renal Plasma Flow the Glomerular Filtration the Excretion and Reabsorption of Phosphorus and the Plasma Level of Calcium and Phosphorus in a Dog

The arrow indicates the intravenous administration of 100 units of parathyroid extract

tubular phosphate reabsorption.[90] The hemodynamic effect after the intravenous injection of parathyroid extract is a rather startling and dramatic one. There are very few procedures indeed which raise glomerular filtration. The familiar phosphate diuresis which for a long time has been known to occur after the administration of parathyroid extract now seems to us to be the result of this increased glomerular filtration rather than to alterations in a mechanism relating to phosphate metabolism. In consequence we have sought to establish whether or not the two effects were induced by a single agent in the material which was being given.

Because we happened to be interested in renal hypertension and could conveniently measure the blood pressure of rats, we injected parathyroid extract into such animals and were able to elicit a very impressive hypertension, which required about 30 minutes to develop and which persisted for 30 minutes to an hour. The systolic values increased from about 103 mm. to approximately 160 mm. of mercury.

Shorr: In this last experiment was the parathyroid extract given intravenously?

Handler: No, it was administered intraperitoneally.

Whatever the material is which elicits this effect, it can be removed very simply by dialysis of commercial parathyroid extract. The responsible agent is not identical with that which affects the phosphate reabsorption, the renal plasma flow, or the serum calcium level.[90]

Attempts to Fractionate the Biologic Activities of Parathyroid Hormone into Separate Compounds

We have never successfully separated the material which causes the hemodynamic alteration from that which causes the rise in the serum calcium level or the inhibition of tubular phosphate reabsorption. We believed that the time was right to engage in such studies. In the last ten or fifteen years much has been learned about procedures to separate and purify proteins, and we thought it was time that someone went back to the parathyroid glands and started all over again. We have now employed practically every device which has been used in other studies to separate proteins *with no success whatsoever*. We have data which look excellent. For example, the data which look most impressive and yet which are almost meaningless were obtained by Dowex chromatography. We succeeded in putting the material on the Dowex column in the acid form and then eluted it with buffers of various sorts.

[90]Handler, P., and Cohn, D. V.: Effect of Parathyroid Extract on Renal Function. *Am. J. Physiol.* **169**: 188 (1952).

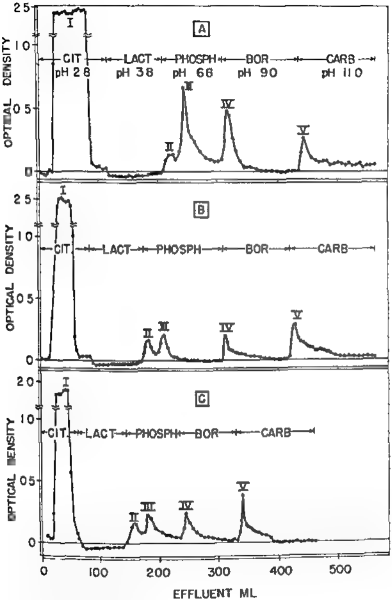

Fig 106 The Chromatographic Analysis of Purified Parathyroid Extract on Dowex Column

The columns employed were Dowex 50 in the sodium form The prote n content f the effluent was measured by it abso pti n at 273 m *Cit* — c trate fraction *Lact* — lactat f action *Phospl* — phosphate fract on *Bor* — borate fracti

The protein concentration of the elutates was measured by the characteristic absorption due to tyrosine in the Beckmann Spectrophotometer. A typical set of results is shown in Figure 106. Here are perfectly clean separations of protein fractions. In no case was the eluting buffer changed until it had removed all the protein that was possible. Each fraction was then assayed for its ability to raise the serum calcium concentration in the dog (that is, by the Pharmacopoeia assay) and the results were expressed as units per milligram of nitrogen. But the activity so expressed proved to be essentially the same in all fractions! This procedure required a great many assays, a large number of dogs, and a considerable quantity of parathyroid glands from Armour and Co.[2,3] This same lack of concentration or separation of biologic activity also has been obtained in simple countercurrent distribution studies; these data are questionable, however, as it was difficult to find suitable sets of solvents to operate the system.

The possibilities which we can suggest at the moment are: 1) As in the case of the adrenocorticotropic hormone, the active material in the gland actually may be a large protein which, in the course of the isolation, is degraded into fractions of varying size, each of which still has activity. 2) The active material may not be a large molecule at all, but instead a small molecule which adheres to each one of these fractions. It is rather surprising, if this is so, that the small molecule elutes cleanly with each one of them; nevertheless, this is an alternative possibility.

We are trying other approaches now, but none of them thus far has come to anything. We have carried out simple procedures such as digesting the active material enzymatically with various proteolytic enzymes. In every case in which we have succeeded in destroying the ability of the material to raise the serum calcium level of the dog, we have lost also the ability to increase the glomerular filtrate rate of the animal. Whether this effect on renal hemodynamics reflects any normal physiological property of the material which emerges from the parathyroid gland normally, I do not know. It is true that the phosphate diuresis and the rise in the glomerular filtration rate are much more dramatic when the experiments are performed in parathyroidectomized animals that are maintained on calcium than when they are carried out in normal animals.

The literature which we have examined carefully is replete with data indicating the same phenomenon — a relatively trivial (and ordinarily

---

Carb — carbonate fraction. The material shown as emerging from the citrate fraction in *A* constitutes the initial breakthrough. This was applied to a second column with the results shown in *B*. The breakthrough from the second column was applied to a third column with the results shown in *C*.

[Unpublished data of Cohn, D. V., and Handler, P.]

perhaps ns gn ficant bu nevertheless real) d m nut o the g o meru ar filtrat on rate after parathyro dectomy The marked phosphatur a wl h has been obser ed so frequently when h poparathyro d pat erts are g en parath ro d extract I suspect at least n part due to the d ure a d the ncreased glomerular filtrat on rate of phosphate rather than metabol c effect on the k dney That d ures a o ca produce n rea ed phosphatur a by the way we have ob er ed n other exper men h h *were not performed for the purpose ot es gat ng th s pr n In a* stud of the fate of gl ne (wh ch was carr ed out n ollabo at o w tl Dr Henry Kam n) where the a terat ons n ur na y phosphate ex ret on ere fol o ed dur ng large cale nf s ons of g ne the er m phospha e concentrat on went down wh le the ur nar pho phoru exc et on ncreased e finall succeeded n lower ng the se um pho ph ate co ce trat on t 0 f mg per 100 cc (F gure 107) These man fe tat o s o c rre l w thout the adm n strat on of paratl ro d extra t smpl a a con equen e of glyc ne d ure I an certa n therefore that n pa t some of tl e effect h ch l a e been ob er ed in the pa t after the adm n trat on of parathyro d hormone (so called as obta ed commerc all) ha been just the resu t ot d ures and actually ma not represent the norn al man festat ons produced by th s l ormo e

We are cont nu ng these stud es of the parath o d gland and our at temp s to fract onate t w th the hope that e can obta n a substance wl ch mo e clo ely approx mate the act ve mater al

Conference Discuss on

Sl o r Co ld you ay son eth ng about formaldehyde n re a to h s problem

Ha d er I know only what I read a fe v weeks ago n a paper n *Endo crinology* by S ewart and Bowen They treated commerc al paratl ro d extract w th formaldel yde and repo ted that they had nact vated tl e n a ter al w th re pe t to ts ab ty to ra e the serum calc um of the dog w tl out affect ng ts ab l ty to cau e pho phat r a It ma vell be that they have nact ated the serum calc um ra ng pr nc ple and perl aps also tl e ab ty of *tl e r mater al to nl b t the tul ular reab orpt o of pl ospl a e* I suspect that tl e l emod nan c effect goe on nchanged un ler tl o e c cum tances becau e fore gn prote n n general also can el c t such an effect

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Hand P Kam n H and Ha J S Tl e Me ab l m of Pa en e ally Ad m n e ed Am o Ac ds I Glyc ne *J B I Cl* 179 63 (1949)

S wa G S and Bo n H F The U na y P o p a e Ex e on Fa to f Pa a y o d G and Ex a t A Ho mo e or an Ar efa t *E d n o* 51 80 86 (1)

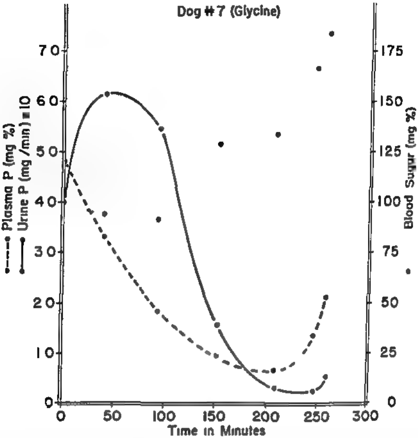

Fig 107 The Effects of a Constant Infusion of a Solution of Glycine in an Anesthetized Dog

The solution contained 4 grams of glycine in 0 3 per cent sodium chloride and was given at the rate of 0 37 ml /kg /min the dog weighed 14 kilograms and was under Dial anesthesia Note that the flow of urine was of the same order of magnitude as the rate of infusion after the first 90 minute Note also the sweeping out effect on phosphate and the remarkably low concentration of serum phosphorus that was found Similar results were obtained by Na_2SO_4 diuresis [2 1]

[This figure is similar to one given elsewhere but has not been published previously]

If that be the case then these investigators h succeeded in demonstrating that the hemodynam iple of does not require pre cisely the same ture as calcium raising or phosphate reabsr s) f Stewart and Bowen most definitely s evid aterial which rai es

the serum calcium level is different from that which inhibits the tubular phosphate reabsorption Their criterion phosphaturia was much too crude since we have shown some time ago that such extracts as they used may elicit phosphaturia by two entirely different mechanisms

Incidentally our work has been started with raw parathyroid glands because our yields were somewhat better when we used the glands instead of commercial extract

Armstrong I was very much impressed by your finding that the active principle was associated with at least three different protein fractions

Handler No not just three fractions but perhaps an infinite number of fractions

Armstrong But you obtained three

Handler We found three pharmacologically different properties Two of these are associated with five fractions obtained by Dowex chromatography

Armstrong Are the fractions continuously distributed over the column or are the fractions separated?

Handler This is partition chromatography at first everything is on top of the column then you elute with buffers of increasing pH and for each one you get a peak of material from the column When no more nitrogen comes out of the column then you change the pH of the eluting fluid and you get another peak and so on We collected five fractions I am certain that we could double that number by narrowing the pH ranges

Armstrong You indicated that the active substance might be somethin that just goes along in association with the proteins

Handler Yes with each of them

Armstrong It seems to me that that is a very good idea

Handler If so it is rather unusual because it means that whatever this material is that is coming off it is a substance which cannot itself be fractionated by partition chromatography That is it is a substance which is totally insensitive to the pH of the buffer that is being used to remove it and in some way (we have been using the word bound for two days now and I can use it perhaps for the last time) bound to protein or proteinaceous materials

Follis Do the proteins that come off the column have molecules of about the same size or of different sizes?

Handler No each one of these fractions was heterogeneous when put into the ultracentrifuge Each fraction was obtained by virtue of its acidic products not because it represented unique molecular species as it were We have no single species in these fractions We have tried in addition the trick of ultracentrifuging and removing several fractions as we ultracentrifuged the material These fractions also had exactly the same amount of biologic activity per milligram of nitrogen

Armstrong Have you tried alkaline hydrolysis?

Handler Yes If you carry out alkaline hydrolysis to a sufficient degree you destroy everything If you carry it out less intensively I do not know what happens

THE EFFECT OF INTRAVENOUS PARATHYROID EXTRACT ON A PARATHYROIDECTOMIZED DOG

FREDERIC C BARTTER

From the National Heart Institute Bethesda Maryland and the United States Public Health Service Hospital Baltimore Maryland

Armstrong Dr Bartter will you please present your data

Bartter The one difference that I have noticed between the phosphate metabolism of anesthetized and of unanesthetized dogs is in the maximum tubular reabsorption of phosphate It is clearly lower under anesthesia

I want to retain for us a ray of hope that parathyroid extract given intravenously may not always be heterogeneous in its action , Figure 108 shows the results of a study on a parathyroidectomized dog

During the control period there was almost no phosphorus in the urine Parathyroid extract was given intravenously (100 units over a 3 minute period followed by a sustainer of 5 units a minute) The sequelae were 1) a very slight rise in the glomerular filtration rate (the amount of phosphorus filtered never significantly exceeded that in the control period) 2) a tenfold rise in the urinary phosphorus excretion and 3) a fall in th serum phosphorus level The urinary phosphorus excretion even after the tenfold increase was only a small fraction of the amount filtered but the increase was sufficient to produce a fall in the serum phosphorus level from 3 5 to 2 2 mg per 100 cc

Conference Discussion

Butler This is a good demonstration of the effect of parathyroid in decreasing the ratio tubular resorbed phosphate/glomerular filtered phosphate (TRP/GFP)

Bartter At least it shows an increased excretion due entirely to failure of reabsorption The ratio TRP/GFP throws no light on mechanism It would fall whether the parathyroid extract produced phosphaturia by decreasing the reabsorption of phosphate by increasing the filtration of phosphate or by both mechanisms

Handler How consistently do the sequelae you have reported occur?

Bartter I do not have a great many experiments It happens consist

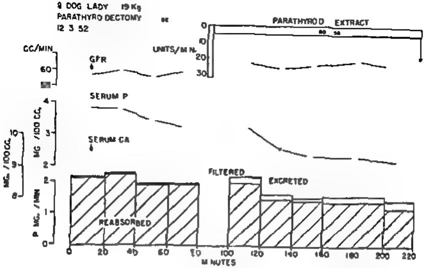

Fig 108 The Glomerular Filtration Rate the Serum Inorganic Phosphorus Levels the Serum Calcium Levels the Urinary Phosphorus Excretion and the Calculated Amounts of Phosphorus Filtered and Reabsorbed Before and During the Intravenous Administration of Parathyroid Extract to a Parathyroidectomized Dog

G F R—glomerular filtration rate The parathyroid extract was given intravenously in a dosage of 100 units *over a 3 minute period followed by a sustaining dose of* 5 units per minute The calculated amount of phosphorus filtered per minute is represented by *the total height of the columns* the amount that appeared in the urine is plotted downward *(the clear area)* from the top of the columns Thus the amount reabsorbed appears as *the hatched area in the bottom of the columns*

ently in this dog I suppose it has something to do with the particular batch of parathyroid extract

Handler Yes it might

Butler I note that Dr Bartter used Lilly extract Did you use Armour preparations Dr Handler?

Handler We used Lilly extract but Armour glands The glands were excellent and we made the protein mixture from them However our initial observations of these differences were all made with Lilly extract which also contains an assortment of proteins and polypeptides

Sobel Where does dihydrotachysterol fit into this picture?

Harrison If you are interested I have some charts showing how vitamin D compares with parathyroid extract in the human being

A COMPARISON OF VITAMIN D AND PARATHY ROID EXTRACT IN MAN [93]

HAROLD E HARRISON and ROBERT KLEIN

Fro tl e Balt o e City Hosp t l Palt ore Mar la l

Ar s ro g Dr Harr son tell us al out our o e vat o s

Harr ■ These stud es vere ı ade on a boy v th d opath c hypopara th ro d sm F gure 109 sl o s tl e rate of the ar pho phate exc et o at d fferent level of tl e serum pl ospl ate before treatment fol o v ng the first ect on of p ratl yro d extract and then after the adm n trat on of large loses of tan D The ser m phosphorus le e were n reased graduall n each study l y tl e ntra enous nject o of an 0 1M solut on of sod um pl osphate at pH 7 4 Tl e excret on rate at any le el of erun pl ospl orus s h gher follow ng the adm n trat on of parathyro d extract or tam n D ■ vould b expected The mechan ■ by wl ch tl ncrease s produced l y these two sub tances appears to be d fferent l o e er wh cl n ay answe Dr So el s uest o The fi d ng a e llu trated n F gures 110 and 111

In F gu e 110 and 111 he p o phate load p e e ed to he tubules e he pho pha e fi ed th ough the g ome u plotted *as th pp po n of acl t)* ga n e concent a n of the se um pho pha e The pho pha e ex e ed n the u ne s p ot ed s m a l *as le l u po of e s) T l n h of he u al l e n g ea s of u o po* (re esen g l e plo of the pho pha e load a d he plot of the u na y phospha e ex et on s he e ma ed amount of tubu a reab p n of p o pha e In the e expe m n s the end genous c ea n ne clea an e was u d as a measu e of t e glome u r fil a o a e and he pho phate load was ca u a ed by n u p ng the ea n ne clea an e b l e se um pho pho u value

In F gure 110 the pho phate loads at any level of ser m phosphorus (tl e pper p o nts) are approx mately the sane before and after tl e njec t on of the paratl yro d extract S nce the rate of excret o of pl o phate ■ ncreased after the parath ro d extract adm n trat on (e tl e lo er po nts are h gl er tl an tho ■ before therapy) tl e tubular reab orpt on of pl o pl ate s lecreased after the njec on of tl e parath ro d extract

In F gure 111 sl o n a s m lar stud fo low the a lm n strat on of v tan n D Tl e surpr ng find ng tl e marked ncrea e n tl e glomerular fi trat on rate after treat ent th v tam n D so tl at the pho pl ate load

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The e s ud e w e s ppo ed by gran s f o le Nu on F u da In a d le Playtex Pa k Pe ea ch Fou d o

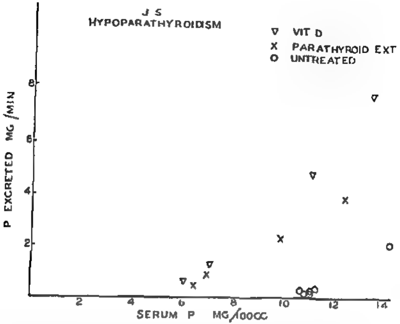
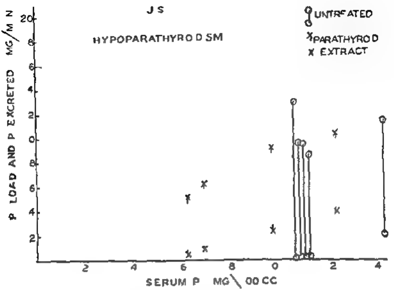


Fig 109 The Effect of Parathyroid Extract and of Vitamin D on the Rate of Excretion of Phosphorus in the Urine at Various Levels of Phosphorus in the Serum of a Child with Idiopathic Hypoparathyroidism

The open circles represent the values before treatment *the crosses* represent the values following parathyroid extract therapy (10 cc per day for 4 days) and *the open triangles* represent the values following vitamin D therapy (150 000 units per day for 10 days) The serum phosphorus levels were increased gradually in each portion of the study by the intravenous injection of a 0 1 M solution of sodium phosphate at pH 7 4

(the upper points) are greatly increased The urinary excretion of phosphate is increased following vitamin D therapy (*i e* the lower points are higher than before therapy) and this is due to the increased phosphate load so that the renal tubular reabsorption of phosphate is as high as in the untreated state

The end result of parathyroid extract or of vitamin D administration upon the urinary excretion of phosphate is the same but the mechanism whereby the increased excretion is produced appears to be different We have repeated these studies in another child with hypoparathyroidism with similar results These experiments were made in collaboration with Dr Robert Klein who is now at Pittsburgh He has independently studied a patient with hypoparathyroidism using inulin clearance as a measure of filtration rate and also by this procedure has found an increase in the glo-



F g 110 The Effect of Para hyro d Extract on tl e Renal Excret on ot Phospl ate of a Ch ld w h Id opatl c H poparathyro d sm

T op ep e he a u s befo e ea m n s p e en he a ue fo ow n pa a hy o d ex ac he apy (10 c pe da fo 4 days) upp po rep e the ph p a e load a ea h nd a ed le e of the e um pho pho us h ou po n ep e en the u na y ex e on of pho pho us a e h nd a d le el of he um ph ph u and e ng h of ica l o ng upp and p n s ep e en s he e ma ed am un of pho pha e eab bed by he ena ulu e a ea h nd ca ed e e of he e un pho p u T e se um pho pho us e e s we e n eased g adua n ea h po on of he ud b the a enous n on of a 0 1 M o u n of sod um pho a e a pH 7 4 The pho pha e load wa a u a ed f om he fo w g fo mu a

$$\text{P l ad } \quad \frac{\text{serum P} \quad g\ 100 \quad) \times \text{G F R}}{100}$$

GFR g me u a a on a e

merular filtrat on rate of phospl ate after tam n D was g en

Conference D scuss on

Ha dlcr The d stance bet veen the upper and the lo ver po nts repre sents phosphate h ch has been reab orbed

Harriso The upper po nt represents e calculated pl osphate load and the length of the l ne nd cates the tubular reabsorpt on of pho phate

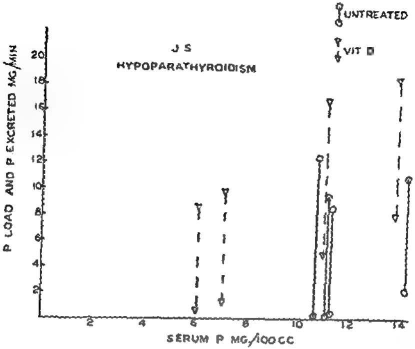


Fig 111 The Effect of Vitamin D on the Renal Excretion of Phosphate of a Child with Idiopathic Hypoparathyroidism

The open circles represent the values before treatment *the open triangles* represent the values following vitamin D therapy (150 000 units per day for 10 days) *the upper points* represent the phosphate load at each indicated level of the serum phosphorus *the lower points* represent the urinary excretion of phosphorus at each indicated level of the serum phosphorus and *the length of the vertical lines connecting the upper and the lower points* represents the estimated amount of phosphate reabsorbed by the renal tubules at each indicated level of the serum phosphorus The serum phosphorus levels were increased gradually in each portion of the study by the intravenous injection of a *0 1 M* solution of sodium phosphate at *pH 7 4* The phosphate load was calculated from the formula given in Fig 110

Handler I do not see what looks to me like a difference in Figure 110

Harrison I think that there is a difference The phosphate load is about the same before and after parathyroid extract administration but the tubular reabsorption of phosphate is less after parathyroid extract injection

Handler Oh I see! I was making the wrong comparison

Shorr Dr Harrison you attribute the increased urinary phosphate excretion after vitamin D administration entirely *to the increased filtration* rate?

Harrison Yes in this particular patient and at this particular time this effect was observed after ten days of vitamin D therapy

Handler Has this effect of vitamin D on the filtration rate been observed previously?

Harrison Not to my knowledge

Handler You had no indication of the renal plasma flow when you were making these studies

Harrison No

Sobel These observations are very interesting Nevertheless I still wish to repeat my question Where does dihydrotachysterol (A T 10) fit into this picture?

Harrison Dihydrotachysterol has the same effect as vitamin D

Sobel You believe it is the same?

Harrison At the dosage level of vitamin D used in the treatment of hypoparathyroidism every effect of dihydrotachysterol can be duplicated by vitamin D Interestingly enough in a case of refractory rickets we observed healing of the rickets when dihydrotachysterol was given in amounts by weight comparable to the amounts of vitamin D that are necessary to produce healing There are differences in the molecular structure of the two compounds of course but in this unphysiological dosage range their effects probably are mediated through the same mechanism

Kramer Parathyroid extract and dihydrotachysterol exhibit the same effect in hypoparathyroidism but not in pseudohypoparathyroidism In the latter condition parathyroid extract has no effect upon the excretion of phosphorus by the kidney but there is a response to dihydrotachysterol

Harrison And also to vitamin D We have not performed experiments such as I have reported with dihydrotachysterol but it is probable that the effects of this compound would be the same

Sobel As I understand it dihydrotachysterol is particularly potent in raising the serum calcium level

Harrison Is it?

Sobel I thought vitamin D and dihydrotachysterol behaved differently

Handler Dr McLean what is your comment on that point?

McLean As far as we were able to find out in our own experimental work and from the literature the effect of dihydrotachysterol and vitamin D in appropriate dose was identical Certainly in the treatment of hypo-

parathyroidism there is no reason to believe that dihydrotachysterol is better than vitamin D Vitamin D got a bad name early because of some of the impurities in vitamin D from over irradiated ergosterol and it was believed for a time that it was much more toxic than dihydrotachysterol but I think there is no evidence at all for that at the present time with the preparations of vitamin D that are now available

Shorr We use vitamin D exclusively

Butler We do too We found that if we used as you say the same milligram dose of vitamin D and of dihydrotachysterol we got the same result But in the last few years we found great variation in the batches of dihydrotachysterol I wonder if anyone else has observed this? We have therefore given up using dihydrotachysterol altogether and use vitamin D instead

Shorr We gave dihydrotachysterol up a long time ago for this very reason

Harrison Calciferol (vitamin D) is so much cheaper than dihydrotachysterol

Shorr There is one point I might add here by way of a warning that is that although one seems to be giving vitamin D in accordance with the daily needs of the patient one often is surprised by the amount of storage that has taken place In patients with pseudohypoparathyroidism and in patients with surgical hypoparathyroidism we have discontinued vitamin D therapy for well over two years and have observed a persistence of normal serum calcium and phosphorus levels even in patients who exhibited in their blood levels during the course of the therapy no evidence that an excess of vitamin D had been given It makes the evaluation of the relative effectiveness of dihydrotachysterol and of vitamin D difficult

Gutman I might say in that connection that the hypercalcemia produced by vitamin D also may persist for a very long time many months after discontinuance of the drug

Bartter Dr Shorr we had an experience similar to yours in Boston We had a patient with very well documented pseudohypoparathyroidism who left our clinic and turned up finally in a mental hospital where she still had normal serum levels although she had not been taking therapy

THE QUESTION OF TWO OR MORE FORMS OF PHOSPHATE IN PLASMA[94]

PHILIP HANDLER and DAVID V COHN[95]

From the Departments of Biochemistry and Nutrition Duke University School of Medicine Durham North Carolina

Armstrong Dr Handler will present some additional studies which are pertinent to the discussion

Handler Most of the discussion this afternoon has concerned the behavior of phosphorus All of the calculations including our own assume that the problem which exists with respect to the serum calcium (*i e* that there are at least two forms) does not hold true for the serum phosphorus and that it is a simple inorganic orthophosphate

As most of you probably know Jean Govaerts a Belgian has published a series of papers indicating that the inorganic orthophosphate of plasma exists in at least two forms one of which is not as readily filterable as is the other This work was done with P^{32} and the essential procedure was to give inorganic P^{32} as orthophosphate and to compare the specific activity of the urine with that of the plasma during consecutive intervals of time He found that the specific activity of the phosphate in the urine was higher than that in the plasma during the same period of time

Parfitt The specific activity is high in the urine I would like to discuss that point

Handler Govaerts observations were absolutely correct The explanation for his unexpected findings (as we discovered after several experiments using his procedure) was that he was ignoring the lag time in the kidney If his data were corrected by about three minutes one could just ignore the whole problem This is shown in Figures 112 and 113 A large dose of P^{32} (in counts per minute not in absolute amounts) was given to *Dog A* Note the Govaerts effect After 100 minutes the plasma and the urine had equilibrated in his terms and by his concept and therefore the two forms of plasma phosphate also had achieved equilibrium At this

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This work has been supported by the U S Atomic Energy Commission under contract AT (40 1) 783 with Duke University

Much of the work was performed during the tenure of Dr Cohn as an Atomic Energy Commission Pre Doctoral Fellow

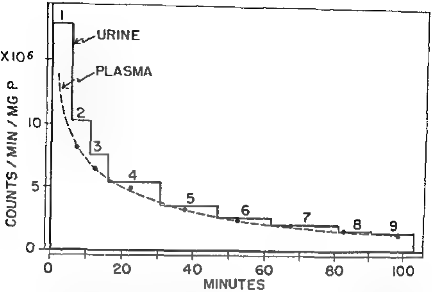

Fig 112 Comparison in Dog A of the Specific Activity of Phosphate in the Urine with That of Phosphate in the Plasma During Consecutive Intervals of Time Following the Intravenous Administration of Radiophosphorus as Inorganic Orthophosphate

At *zero time* 5 *mc of* P^{32} *were given intravenously* as inorganic orthophosphate For 30 minutes the specific activity of the urinary phosphate exceeded that of the plasma phosphate At 98.5 minutes blood was withdrawn for transfusion into *Dog B* (see Fig 113)

[Reproduced by permission from Handler P and Cohn D V Use of Radiophosphorus in Studies of Glomerular Permeability of Plasma Inorganic Phosphate *Am J Physiol* 164 646 (1951)]

point plasma from *Dog A* was injected into *Dog B* Note that the entire phenomenon reappeared! These experiments entailed a single injection of P^{32} therefore from time *zero* the specific activity of the plasma phosphate was falling logarithmically If however a steady infusion of P^{32} was given so that for some time the specific activity of plasma phosphate was rising the results were the converse of those previously found that is the activity *of the phosphate in the plasma exceeded that of the phosphate in the urine* (Figure 114) If a large priming dose is given followed by a continuous infusion which is suddenly stopped the Govaerts phenomenon dramatically reappears (Figure 115) There is little doubt but that this indicates merely the lag time of the kidney The urine that can be collected at any given moment is derived from the plasma that passed through the glomerulus 3 to 5 minutes earlier If this interpretation is valid we can return com

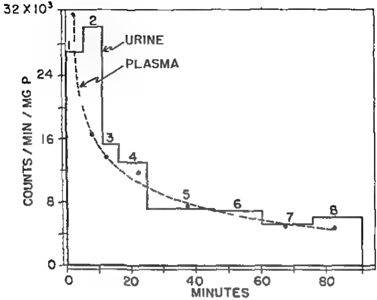

Fig 113 C mparison D g I f the Specific Activity of Phosphate in the Urine with That of Phosphate in the Plasma During C nsecutive Intervals of Time Following Transfusion with Radi phosphorus Containing Plasma from D g A (Fig 112)

At zer t me 10 cc of plasma from *D* *A* (see Fig 112) ta g 74?000 u per m ut er jected t a enously n *Dog B* Note the r ppearance of the d crepancy between the specific activity of the urinary p o plate a d that of the plasma phosphate If the data Fig 112 reflected equilibration of t o f rms of plasma phosphate then the data Fig 113 should ot have shown the same effect ce the two or re f rms already would have been equilibrated n the administered plasma

[Reproduced by perm fr m Handler P a d Cohn D V U of Radi phosphorus n Studies f Glom lar Permeability of Plasma I ganic Phosphate *A J Ph s l* 164 646 (1951)]

t rtally t r r form that plasma r g m ortho phosphate s just that and n th gel c [20]

Further Dr Stanley Bradley did the same type f experiment with a number of labeled substances and found that there was a rather incredibly

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[20] Handler P a d Cohn D V Use of Radiophosphorus n Stud s of Gl merular Permeability of Plasma Inorganic Phosphate *A J Physiol* 164 646 (1951)

Bradley S L Nickel J F and Le fer E The D t bution of Neph n F n t n Man *Trans Asso A n Physic a s* 65 147 158 (195)

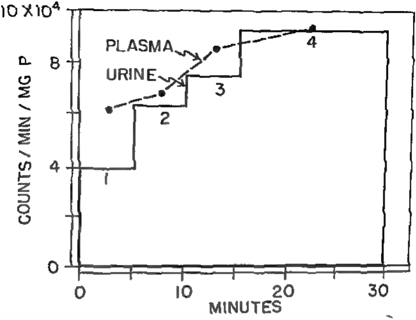

Fig 114 Comparison in a Dog of the Specific Activity of Phosphate in the Urine with That of Phosphate in the Plasma when Radiophosphorus Is Given so that the Specific Activity of the Phosphate in the Plasma Is *Rising Throughout the Period Studied*

At zero time the animal was given a priming dose of radiophosphorus intravenously and this was followed immediately by a constant infusion of radiophosphorus through out the period studied Note that the renal lag time results in a reverse Govaerts Phenomenon

[Reproduced by permission from Handler P and Cohn D V Use of Radio phosphorus in Studies of Glomerular Permeability of Plasma Inorganic Phosphate *Am J Physiol* 164 646 (1951)]

high lag period of the order of 45 minutes before the specific activity of the urine fell to that of the plasma (when the latter itself was falling) Similar results were obtained with sodium potassium urea and (using certain assumptions) inulin Presumably the dead space of the kidney was supplying over all of this time urine higher in active material than the plasma

Armstrong The same worker with radiocalcium did not find the same selection between calcium in the urine and in the plasma I have never been able to understand his experiment because he would obtain 10 or 15 cc of urine per minute from a small dog

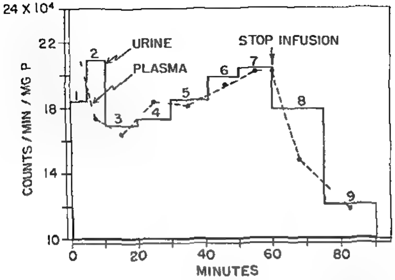

Fig 115 Comparison in a Dog of the Specific Activity of Phosphate in the Urine with That of Phosphate in the Plasma when Radiophosphorus Is Given so that the Specific Activity of the Phosphate in the Plasma Is Both Rising and Falling during the Period Studied

At zero time the animal was given a single dose of radiophosphorus intravenously After an interval of 15 minutes without injections the animal was given a constant infusion of radiophosphorus for 45 minutes At 60 minutes after the start of the experiment the *infusion was discontinued but the observations were continued* for another 30 minutes

[Reproduced by permission from Handler P and Cohn D V Use of Radiophosphorus in Studies of Glomerular Permeability of Plasma Inorganic Phosphate *Am J Physiol* 164 646 (1951)]

Shorr How small a dog?

Armstrong Five or eight kilograms

APPLICATIONS OF CHELATING AGENTS[298]

MARTIN RUBIN

From the Chemo Medical Research Institute Georgetown University Washington District of Columbia

Armstrong Dr Rubin tell us about the binding of metals by chelating agents

Rubin · The name chelate derives from the Greek chela = claw The chelating agents under discussion today function as claws in holding on to cations in solution Metals bound in this way are in the form of soluble undissociated physiologically unavailable complexes

The Nature of the Combination of Chelating Agents with Metallic Cations

The nature of the combination of a typical chelating agent ethylenediaminetetraacetate[299] (EDTA Versene or Versene Regular) with a typical cation calcium is indicated in Figure 116 Calcium is bound in this structure not only by the ordinary ionic valences to the carboxyl groups but also by secondary valence bonds to the nitrogen atoms While this is an example of a relatively weak chelate the calcium ion in equilibrium with this structure is less than that in equilibrium with calcium oxalate In other words calcium would not be precipitated from EDTA solution with oxalate

Other types of chelate structures are known as may be illustrated in Figure 117 The strength of the binding of the metal in these combinations is a function of both the structure of the organic component in the system as well as of the particular metal under consideration The metal chelate bond strengths are of the greatest significance in attempts directed toward biological or therapeutic applications of this group of materials A relative order of the strength of combination of EDTA for some cations is given in Table XXXIV It should be noted that the metals highest on

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[298]These studies were supported by a research grant from The Bersworth Chemical Co Framingham Massachusetts

[299]Trade name for ethylenediaminetetraacetate The Bersworth Chemical Co Framingham Massachusetts

Martell A and Calvin M *The Chemistry of Metal Chelate Compounds* Prentice Hall New York (1952)

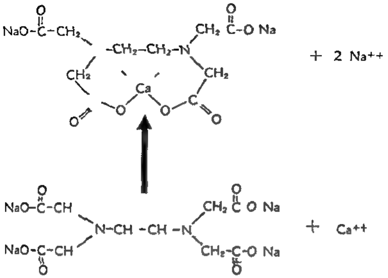

Fig 116 Versene Calcium Chelate

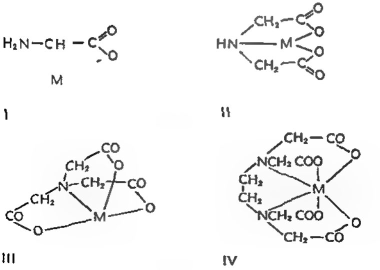

Fig 117 Types of Metal Chelate Structures

TABLE XXXIV

The Relative Order of Cation Combinations with Ethylenediaminetetraacetic Acid* (EDTA)

Chromium	Calcium
Copper	Magnesium
Nickel	Strontium
Lead	Barium
Cobalt	Radium
Calcium	

*Versene Regular

the list will displace those below them from a combination with this chelating agent

Biologic Application of Calcium Binding Chelating Agents

When chelating agent are introduced into the physiological environment their primary gross action is on the divalent cation present in greatest degree namely calcium A detailed discussion of these effects is given in the next presentation However as a consequence of this calcium combining action certain other therapeutic possibilities have been developed which can be mentioned The dissolution of calcium and magnesium urinary calculi has been described[301, 30] The use of EDTA as an in vitro anti coagulant for the preservation of whole blood recently has attracted considerable attention [3 3 0] In a promising study calcific opacities in the eye

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[301]a Raymond S and Gehres R F Ethylenedinitrilotetraacetic Acid as Solvent for Urinary Calculi *Proc Soc Exper Biol and Med* 74 715 (1950)
b Gehres R F and Raymond S New Chemical Approach to Dissolution of Urinary Calculi *J Urol* 65 474 (1951)

[0] Abeshouse B S and Weinberg T Experimental Study of Solvent Action of Versene on Urinary Calculi *J Urol* 65 316 (1951)

[1 3] Dyckerhoff H Marx R and Bayerle H Comparative Studies of the Biology of Conserved Blood *Z ges Exp Med* 113 291 (1944)

[30] Klapheke M A and Kubin M Sodium Ethylenediaminetetraacetate as an Anticoagulant for Routine Laboratory Procedures *The Bulletin Georgetown University Medical Center* 5 33 (1951)

[305] Proescher F Anti coagulant Properties of Ethylenebis iminodiacetic Acid *Proc Soc Exper Biol and Med* 76 619 (1951)

[06] Dillard G H L Brecher G and Cronkite E P Separation Concentration and Transfusion of Platelets *Proc Soc Exper Biol and Med* 78 796 (1951)

have been dissolved by irrigation with EDTA solution [7] In addition to these possible therapeutic applications of the calcium combining action of the chelating agents this property has been utilized as a means of preparing bone for histological study [8, 9]

The injection of the preformed metal chelates of a lower order of stability than the calcium complex results in displacement of the cation by calcium according to the equation $MeV + Ca^{++} \rightarrow CaV + Me^{++}$ Thus when the magnesium EDTA chelate was administered to rabbits dogs or humans there occurred liberation of the magnesium ion simultaneously with a lowering of the systemic calcium levels (Table XXXV) This unbalancing of the calcium/magnesium ratio by simultaneously raising the magnesium level and lowering the calcium level resulted in marked enhancement of the pharmacological activity of magnesium *in vivo* The known hypotensive action of magnesium in high dosage was amplified and hypotensive effects of clinical significance were shown to occur under these conditions (Figure 118) [10]

TABLE XXXV

The Displacement by Calcium of Magnesium in Combination with Ethylenediaminetetraacetic Acid* (EDTA)

Serum		
Magnesium	Calcium	Phosphorus
(mg/100 cc)	*(mg/100 cc)*	*(mg/100 cc)*
1.70	13.90	5.2
Infusion of Magnesium EDTA†		
6.60	11.30	5.2
10.56	6.10	4.4

*Versene

†Magnesium Versenate

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[7] Grant W M (Harvard Medical School) Personal communication

[8] Sreebny L M and Nikiforuk G Demineralization of Hard Tissue by Organic Chelating Agents *Science* 113 60 (1951)

[9] Birge E A and Imhoff C F Versenate as a Decalcifying Agent for Bone *Am J Clin Path* 22 192 (1952)

[10] Popovici A Geschickter C F and Rubin M The Treatment of Essential Hypertension by Magnesium Chelate Solution *The Bulletin Georgetown University Medical Center* 5 108 116 (1951)

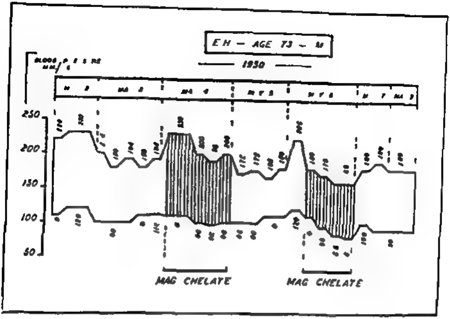

Fig. 118. The Hypotensive Action of Magnesium Versenate in Man.

Chelating Substances as Therapeutic Agents for Intoxications with Metals Such as Lead

Application of the reverse displacement reaction $CaV + Me^{++} \rightarrow MeV + Ca^{++}$ has allowed the utilization of the physiologically inert calcium EDTA complex (calcium Versenate) as a means of mobilizing and excreting toxic metals.[311] Thus in the case of experimental and of clinical lead intoxication the exchange reaction of lead for calcium occurred *in vivo* and resulted in the marked elimination in the urine of the lead EDTA chelate (*Figure 119*).[31]

Handler: How much calcium was administered in this way?

Rubin: In this case a half gram a day of the calcium chelate over a period of five days, then two days without medication, then five days of calcium chelate, then two days without medication, and so on. This would correspond to about 50 mg./day of calcium.

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311 Rubin, M., Gignac, S., and Popovici, A.: *Abstracts of Spring Meeting Am. Chem. Soc.*, Milwaukee, Wisconsin, p. 31 (1952).

31 Bessman, S. P., Ried, H., and Rubin, M.: Treatment of Lead Encephalopathy with Calcium Disodium Versenate. Report of a Case. *Med. Ann. District of Columbia* 21: 312-315 (1952).

Follis Given subcutaneously?

Rubin In this case it was given intravenously Our experience indicates that it can be given orally subcutaneously or intramuscularly with the same result

Follis You say that the calcium Versenate if given by mouth would not be absorbed?

Rubin That is exactly right

Follis If you want to get rid of the lead would you have to give calcium Versenate parenterally?

Rubin No you can give it orally according to Dr Sidbury of Atlanta Georgia in which case the lead in the blood presumably gets into the gastrointestinal tract is picked up by the Versenate and is excreted In that way one maintains a continuous clearance of lead from the blood which in a very remarkably short time removes all of the lead in the soft tissue

Cutman There is no precipitation of acute lead colic as a result of this rapid mobilization of lead?

Rubin None at all The lead under these circumstances is completely soluble it is excreted *in toto* This procedure seems to be an excellent method for treating lead poisoning

Kramer Is there no danger in mobilizing lead from the tissues?

Rubin No because the lead in combination is without toxicity The lead Versenate for example is so non toxic that we have used it as an opaque contrast agent for diagnostic x ray work

Kramer Does the concentration of lead occur in the gastrointestinal tract when the calcium Versenate is given orally?

Rubin The lead Versenate complex when given by mouth is rapidly absorbed and excreted in the urine

Kramer But the flow of lead is from the tissues to the intestine During this process is there any danger from an acute rise in the lead level in the blood

Rubin No because the lead presumably is complexed in the intestine and the blood lead level never rises higher than the level that it is ordinarily in these patients

Kramer That is the question—how high does the blood lead level actually get?

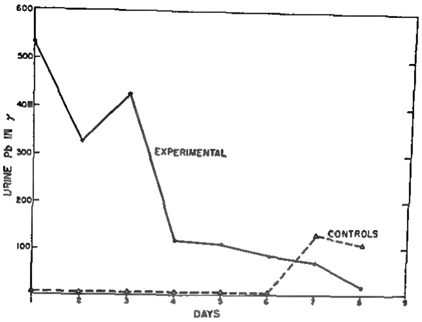

Fig 119 The Urinary Excretion of Lead Induced by the Intravenous Administration of Calcium Versenate

Robinson Once you have cleaned the lead out of the tissues do you have another equilibration of lead between the soft tissues and the bone so that you have to repeat the treatment at a later date?

Rubin Apparently not in the patients studied to date In rabbits however repeated mobilization of large doses of intravenously administered lead by treatment with calcium Versenate causes equilibration between the urine and the bone

In future studies we plan to use the decalcifying action of the non complexed compound to clean out the bone simultaneously We have also done this in animals but it has not yet been tried in humans I think it will be attempted in the light of further work on calcium metabolism which I shall report later

Chelating Agents and Iron

Shorr I know that you have done work on iron Dr Rubin are you ready to talk about it?

Rubin Dr Foreman[1] at Los Alamos has reported on his work with tagged iron We are working with animals studying the effect of chelating agents on iron metabolism Thus far these chelating substances have not been used clinically For combination with iron one uses a different complexing agent for which the pharmacology is not completely known There is much work to be done on this problem if anyone wishes to do it

Armstrong Is this the Sequestrene that I see advertised in *Science* ?

Rubin The same compound is called Sequestrene or Versene How ever there is a whole family of other amino acids which have a selective affinity *for different cations*

Armstrong But I understand that the manufacturers have a substance that is supposed to be highly selective for iron

Rubin It is called Versene Fe 3 Specific

Chelating Agents and Radioactive Metals

Similar results have been obtained in the removal of plutonium and mercury from man (Figures 120 121)[313 4] and of cobalt and nickel from other species[315] In an analogous manner the injection of a chelating agent with strong complexing properties for iron has been shown to result in marked excretion of radioactive iron (Figure 122) Depending on the interval between the administration of a metal and the treatment with calcium EDTA *the extent of tissue fixation and even the distribution* between tissues may be controlled Thus the excretion of radioactive yttrium in rats has been altered (as indicated in Table XXXVI) as a function of the time relationship between the injected radioisotope and the therapeutic agent[317] In human beings the retention of radioactive lanthanum has been controlled in a similar manner (Figure 123) These experiments raise the possibility of the more intensive ultilization of certain

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[1] Foreman H (University of California Los Alamos New Mexico) Personal communications

[313] Sidbury J (Grady Hospital Atlanta Georgia) Personal communication

[315] Rubin M and Gignac S Personal communication

[31] Foreman H Huff R L Oda J M and Garcia J Use of a Chelating Agent for Accelerating Excretion of Radio-Iron *Proc Soc Exper Biol and Med* 79 520 (1952)

[317] Cohn S (U S Radiological Defense Lab San Francisco California) Personal communication

[318] Hart H and Laszlo D (Montefiore Hospital New York City) Personal communication

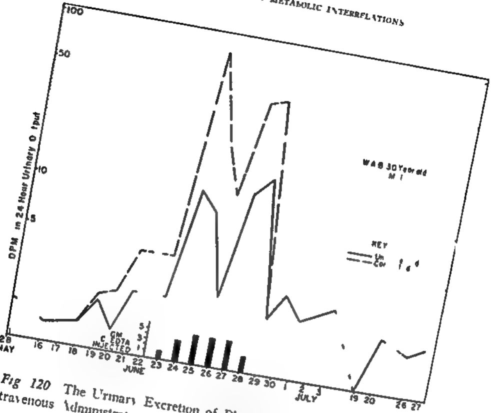

Fig 120 The Urinary Excretion of Plutonium by Man Following the Intravenous Administration of Calcium Versenate

TABLE XXXVI

The Control of Y^{91} Biological Half Life and Tissue Distribution by Calcium Ethylenediaminetetraacetic Acid* (EDTA) Administration

	Excretion	Skeleton	Soft Tissues
Control	29 3	52 8	17 6
Calcium EDTA—after 2 wk	52 7	47 4	9 7
Calcium EDTA—after 1 hr	79 7	17 9	2 4
Calcium EDTA—pretreated	93 0	3 9	2 9

*Calcium Versenate

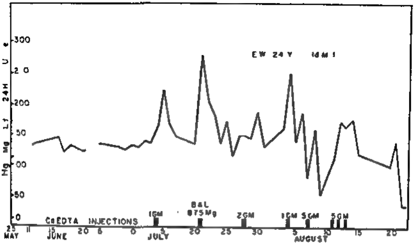

Fig 121 The Urinary Excretion of Mercury by Man Following the Intravenous Administration of Calcium Versenate

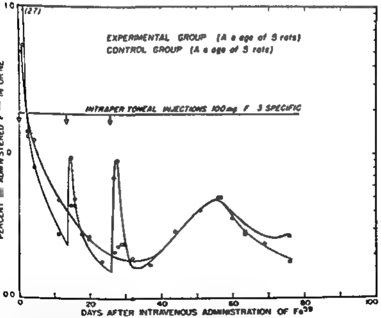

Fig 122 The Urinary Excretion of Radioactive Iron by Rats Following the Intraperitoneal Injection of Versene Fe 3 Specific

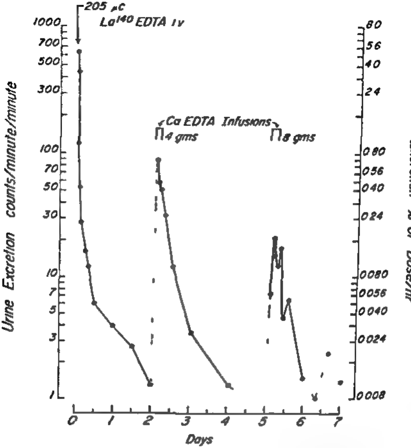

Fig 123 The Urinary Excretion of Radioactive Lanthanum by Man Following the Intravenous Administration of Calcium Versenate

isotopes at higher radioactivity levels for the purpose of producing internal radiation therapy

This brief survey gives some indication of the range of applicability of the synthetic chelating agents

CHELATING AGENTS IN THE STUDY OF CALCIUM METABOLISM

MARTIN RUBIN

From the Chemo Medical Research Institute Georgetown University Washington District of Columbia

Armstrong Dr Rubin will now tell us more of the use of chelating agents in the investigation of calcium metabolism

Rubin It has been mentioned in the previous presentation that the introduction of ethylenediaminetetraacetic acid (EDTA Versene or Versene Regular) into a physiological environment results in the combination of this substance with the circulating calcium[1,*] Measurement of the extent of this combination is facilitated by the failure of the chelate bound calcium to be precipitated by oxalate and hence the changes in the calcium level as usually measured yield an index of the degree of calcium binding by EDTA

Factors Affecting the Degree of Calcium Binding by the Chelating Agent Ethylenediaminetetraacetic Acid (EDTA Versene)

Depression of the physiologically available serum calcium levels has been found to be a function of the route of administration as well as of the rapidity of administration of the chelating agent (Figure 124) Rapid intravenous administration of EDTA to rabbits resulted in an immediate lowering of the effective serum calcium levels with death as a consequence from hypocalcemic tetany Intraperitoneal intramuscular or subcutaneous administration of the compound was followed by less rapid changes in the circulating calcium levels Continued intravenous infusion of Versene in rabbits and dogs resulted in a progressive decrease in the serum calcium levels when the rate of infusion of the calcium combining agent was more rapid than the ability of the skeletal system to replace in the circulating fluids the calcium that was being removed by combination with chelate

Neuman When you say drop the serum calcium level are you speaking of calcium that can be precipitated as an oxalate?

Rubin That is correct The total calcium is unchanged—no that is

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* Popovici A Geschickter C F Reinovsky A and Rubin M Experimental Control of Serum Calcium Levels In Vivo *Proc Soc Exp Biol and Med* 74 415 (1950)

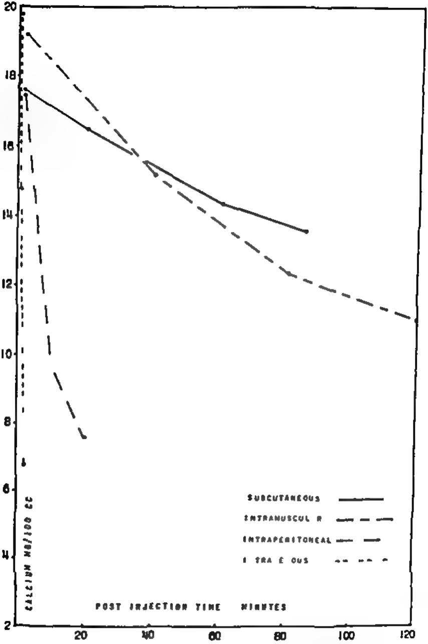

Fig 124 The Effect in Rabbits of Intravenously Administered Versene on the Serum Levels of Physiologically Available Calcium

not entirely true—the total calcium decreases due to the independent excretion of the calcium Versenate complex. However, we are talking at the moment about the oxalate precipitable calcium and the decreases I refer to are in this fraction.

Of interest was the consistent observation that the serum phosphorus levels decreased concomitantly with the fall in the oxalate precipitable calcium.

Species Variation in Calcium Metabolic Mobility

The rapidity of the return to the normal range of the systemic calcium levels after primary hypocalcemia had been induced by the above procedure varied with the species under study. Table XXXVII lists the changes in the calcium levels of the rabbit after intravenous injection of various doses of EDTA. Table XXXVIII presents analogous data for the dog. In Table XXXIX similar results are recorded for the chick from studies to be published by Professor Sturkie and his students at Rutgers University.[3] While these data are not strictly comparable because of the differences in the route of administration, there is a general indication from these and from other studies that the chicken, the rabbit, and the dog, in this order, exhibit an increasing degree of calcium metabolic mobility.

TABLE XXXVII

The Effect on the Systemic Calcium Levels in the Rabbit after the Administration of Ethylenediaminetetraacetic Acid* (EDTA)

Dose	Route	Drop in Calcium	Time Interval
(mg/kg)		(%)	(minutes)
12 5	intravenous	3	3
25	intravenous	46	15
50	intravenous	61	2
100	intravenous	85	2
300	intravenous (infusion)	64	200
100	intraperitoneal	56	8
200	intramuscular	25	42

*Versene Regular

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[3] Sturkie, P. D., and Pollin, D. (Rutgers University, New Brunswick, New Jersey) Personal communication.

TABLE XXXVIII

The Effect on the Systemic Calcium Levels in the Dog after the Intravenous Administration of Ethylenediaminetetraacetic Acid* (EDTA)

Dose	Drop in Calcium	Time Interval
(mg/kg)	(%)	(minutes)
25	33	20
50	48	10
400 (infusion)	30	180

*Versene Regular

TABLE XXXIX

The Response of the Serum Calcium Levels in Chickens to the Intramuscular Injection of Ethylenediaminetetraacetic Acid* (EDTA)

Dose	Result
(mg/kg) 20	very little drop
30	8.6% change within 10 minutes of injection
30	3.7% change within 30 minutes of injection
50	15.2% change within 5 minutes of injection
50	12.9% change within 10 minutes of injection
50	3.7% change within 30 minutes of injection (1 bird)
50	15.7% change within 50 minutes of injection (2 birds)
150	5.9% change within 5 minutes of injection (6 birds)
150	6.3% change within 10 minutes of injection (2 birds)
150	13.8% change within 15 minutes of injection (1 bird)
150	8.7% change within 20 minutes of injection (8 birds)
150	21.7% change within 25 minutes of injection (2 birds)
150	3.0% change within 30 minutes of injection (3 birds)
150	24.5% change within 35 minutes of injection (2 birds)
150	8.3% change within 40 minutes of injection (7 birds)
150	13.0% change within 60 minutes of injection (12 birds)

*Versene Regular

Quantitative Aspects in Man of the Equilibration of Calcium Between Bone and Circulating Fluids

An effort was made to utilize the hypocalcemic properties of EDTA as a means of lowering the systemic calcium levels in patients with hypercalcemia. Table XL, from a study by Dr. Gellhorn and Dr. Sahagian-Edwards[3], records the calcium level changes in a patient after the intravenous infusion of 20 grams of EDTA over a period of 15 minutes. This quantity of Versene is equivalent in combining power to about 2 grams of calcium, or approximately three times the total amount of calcium in the patient's serum. In a period of 15 minutes, therefore, this patient replaced the quantity of circulating calcium three times. It is possible to calculate from these results that this patient was able to replace the circulating calcium from the labile stores in the skeletal system at a rate of 50 mg. of calcium per minute. It may be recalled that two years ago it was reported at this conference that simultaneous exsanguination and transfusion of a dog with calcium-depleted blood could not be carried out fast enough to induce in the animal symptoms of hypocalcemic tetany.[4] If the dog is as efficient as the human in replacing the calcium of the blood, it is clear that it would have been necessary to re-introduce the calcium-depleted blood at a rate of about a liter per minute to have produced symptoms of hypocalcemic tetany.

TABLE XL

The Serum Calcium Level in Man after the Intravenous Administration of 20 Grams of Ethylenediaminetetraacetic Acid* (EDTA)

Time	Calcium
	(mg./100 cc.)
Start	25.0
15 *min.*	13.7
30 *min.*	8.2
1 *hr.*	8.8
3 *hr.*	16.6
24 *hr.*	16.2

*Versene Regular.

[3] Gellhorn, A., and Sahagian-Edwards, A. (Francis Delafield Hospital, New York City): Personal communication.

[4] Hastings, A. B.: Studies on the Effect of Alteration in the Concentration of Calcium in Circulating Fluid on the Mobilization of Calcium. *TRANS. MACY CONFERENCE ON METABOLIC INTERRELATIONS* 3: 38-50 (1951).

The Relationship Between Calcium and Magnesium Homeostasis

It has been known for some time that hypocalcemic tetany may be eliminated by the administration of magnesium salts. An acute experiment of this type is illustrated in Figure 125. Whereas the control animals treated with Versene intravenously exhibited the characteristic drop in the serum calcium levels and consequently tetany, a similar group of animals given a magnesium sulfate infusion simultaneously with the EDTA showed a rapid restoration of the serum calcium levels. These data indicate a direct inter action between calcium and magnesium homeostasis.

The relationship of calcium and magnesium in normal and pathological states recently has been the subject of more extended study. This work has been facilitated by the development in our laboratories of a rapid, convenient and accurate titrimetric procedure for the simultaneous analysis of the serum calcium and magnesium levels.[323] The ratio of the serum

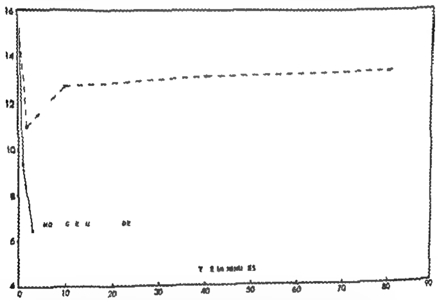

Fig. 125. The Effect of the Simultaneous Intravenous Administration of Magnesium and Versene on the Ability of the Versene to Lower the Serum Level of Physiologically Available Calcium.

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[323]Friedman, H. *The Significance of the Magnesium/Calcium Ratio and a New Method for the Determination of Magnesium and Calcium in Biological Fluids.* Ph.D. Thesis, Graduate Department of Chemistry, Georgetown University (1952).

levels of magnes um/calc um has been fou d to be a sens e nd ator of the metabo c status of these d ale t ons Of nterest tl e find n that a low magne un calc um rat o is u ually the re ult of a lo v erum mag nes um le el and a norn al or h gh erum calc um le el In contra t he le el usually are re ersed when tl e serum magnes n ser m calc um a o s abo normal (Table XLI and XLII)

The Fate of the Intravenously Formed Chelate Complex of Calc um

The quest on may be ra sed as to the fate of the cal m EDTA com p ex that formed after the parenteral adm n strat on of tl s cl elat agent The work of Dr H Fore nan at the Un v rs t of Cal forn a an l at Los Ala nos w tl rad oact ve carbon tagged EDTA estab hed tl at n ra the mater al was rap dl excreted n the u e af er ntra enou

TABLE XLI

The Serum Magnes un and Calc um Values w th a Mg/Ca Rat o Below tl e Normal Range

No	Magnes um*	Calc um*	Rat o*	D agnos
6	*0 28*	5 87	*0 05*	Acute nte t nal ob tru t on
7	*0 8*	5 87	*0 07*	Common du t st ne
8	*0 33*	6 01	*0 06*	Pr or to paracentes s
9	*0 68*	5 59	*0 1*	D abet c coma
10	*1 10*	6 50	*0 17*	Mult p e n eloma
11	*1 03*	5 45	*0 19*	Acute ellow atropl
12	*1 19*	6 43	*0 19*	Mult p e eloma
13	*0 95*	4 89	*0 19*	C rrh s s
14	*1 12*	5 45	*0 21*	Hepa t s nfect ou m o n cleo s
15	*1 20*	5 5	*0 1*	Acute ntest nal obstru t on—after paracen s
16	*1 15*	4 94	*0 23*	Cl ole y t t s—pre operat
17	*1 10*	4 65	*0 24*	Con mon duct stone—post operat e
18	*1 51*	5 21	*0 25*	Cholecyst t s—post operat e

*Va n t n n rmal range a e g n a

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[a] F man H V M and Magee M (Un s y of Ca fo a Los A am N Mex o) Tl e Me ab m of C[14] Lab ed F ct ed am aa e a e I a be p h d

TABLE XLII

The Serum Magnesium and Calcium Values with a Mg/Ca Ratio Above the Normal Range

No	Magnesium*	Calcium*	Ratio*	Diagnosis
75	*3.82*	4.48	*0.85*	Toxemia pregnancy
76	*3.67*	*4.26*	*0.86*	Acute nephritis
77	3.06	*3.54*	0.87	Viral hepatitis
78	*3.84*	*4.31*	0.89	Prepartem edema
79	*4.56*	5.15	0.89	Acute intestinal obstruction
80	3.09	*3.37*	0.92	Pneumonitis
81	2.92	2.98	0.98	Cardiac emphysema
82	*4.17*	4.47	0.93	Acute intestinal obstruction
83	3.31	*3.32*	1.00	Pulmonary congestive heart failure
84	3.05	*3.06*	1.00	Esophageal varices
85	*3.74*	*3.74*	1.00	Cardiac emphysema
86	*3.65*	*3.54*	1.03	Carbon tetrachloride poisoning
87	*3.65*	*3.38*	1.08	Toxemia pregnancy
88	*3.75*	*3.49*	1.08	Hypertension
89	*3.71*	*3.41*	1.09	Diabetes
90	*4.44*	*4.07*	1.10	Acute intestinal obstruction
91	*3.88*	*3.22*	1.20	Cirrhosis
92	*4.57*	*3.49*	1.31	Chronic nephritis
93	*3.92*	*2.87*	1.37	Ulcerative colitis
94	*3.94*	*2.82*	1.40	Peritonitis
95	*4.33*	*2.77*	1.55	Carcinoma of stomach macrocytic anemia
96	*5.80*	*3.57*	1.62	Uremia
97	*5.18*	*3.17*	1.63	Umbilical hernia
98	*6.35*	*3.66*	1.73	Chronic nephritis
99	*3.94*	*2.06*	1.91	Toxemia pregnancy—post partem
100	*3.99*	*1.65*	2.42	Nephritis terminal

*Values not in the normal range are given in italic

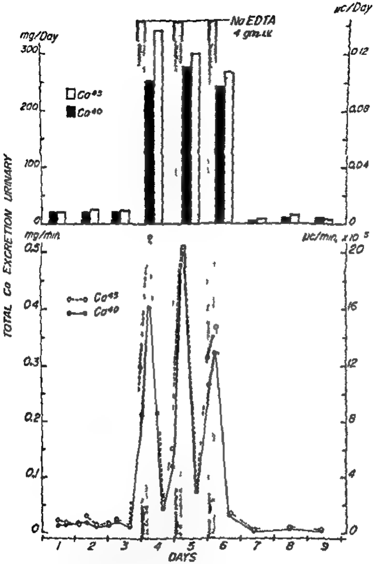

Fig 126 The Urinary Excretion of Radioactive Calcium and of Inert Calcium by Man Following the Intravenous Administration of Ver ene

injection The recovery was quantitative In the human it was demonstrated by the metabolic studies of Dr D Laszlo and his colleagues[3 5] that up to 80 per cent of the calculated amount of complexed calcium was found in the urine within 24 hours (Figure 126) When these studies are analyzed together the evidence indicates that the calcium chelate complex is excreted unchanged as the complex It should be noted that the calcium that was eliminated was excreted in the urine as part of an anionic complex rather than as a cation This shift in the physical state of the calcium resulted in a shunting of the kidney mechanisms for controlling the excretion of calcium The regulating mechanisms which maintained at normal levels the calcium excretion before and after the experimental period did not regulate the excretion of the EDTA chelated calcium

The patient studied by Dr Laszlo and his associates had been pre treated with radiocalcium[45] some days prior to the EDTA administration The fact that the specific activity of the calcium[45] in the urine was maintained during the periods of Versene induced calcium loss proves that the radio active calcium was drawn from the same areas as the non radioactive calcium in the bone lysis induced by the administration of EDTA This experiment supports the view that the maintenance of the systemic calcium levels by the skeletal system is the result of the physicochemical equilibration of the bone area with the circulating fluids

Conference Discussion

Armstrong Can you really tell us what the pharmacological effects are that are produced by the intravenous administration of 20 grams of ethylenediaminetetraacetate? Is it innocuous?

Rubin I would not want to use it indiscriminately Versene has been given a number of times in doses as high as 9 grams without sequelae of any kind In the study of Dr Gellhorn and Dr Sahagian Edwards the dose of 20 grams was discontinued at the point at which the patient began to complain of frontal headache and other uncomfortable effects But note also that these manifestations occurred simultaneously with a serum calcium level of 8 point something a concentration at which you might expect some symptoms to appear

Armstrong Was the material with the C^{14} label given to any human subjects?

Rubin No it was not given to man but to rats The Versene was

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3 Bellin J and Laszlo D (Montefiore Hospital New York City) The Metabolism of Calcium[45] in Humans to be published

radiocarbon methylene labeled Its distribution in terms of calcium therefore was different than that of exchangeable radiocalcium tagged material The compound was given intravenously was detected by its radiocarbon activity and then was identified by paper chromatography as unaltered material Therefore it is completely correct to say that Versene i not utilized biologically

Sobel Do you think that with Versene you might remove essential traces of copper aluminum and zinc?

Rubin We have completed two year toxicity studies at low dosage level and found no striking abnormalities There was some evidence that suggested that there was a derangement in fat metabolism in the rats observed for a two year period This disturbance was indicated by the gross appearance of the animals rather than by histologic or other findings the chemical levels were normal but there was a complete absence of fat in the abdomen Against this interpretation however is the fact that the animal had had a considerable amount of diarrhea and it is known that concomitantly with diarrhea there may be a change in the fat depots similar to that observed in these animals

Storr You employed the oral route of administering Versene in the investigations of toxicity?

Rubin The two year toxicity studies were carried out by oral feeding We have conducted toxicity studies for approximately four months by giving daily injections in rabbits and rats The results were about the same

Henneman In what form did you give the Versene orally? Doesn t it burn the lips? Doesn t Versene burn if you put a drop of it on your tongue?

Rubin The acid yes but the tri sodium salt has a pH in solution of about 7 and is completely innocuous It is mildly anesthetic as a matter of fact We used it at the beach last year for sunburn

Butler How about calcium Versenate?

Rubin Calcium Versenate in contrast to the uncomplexed sodium material is completely inert In Cleveland it has been given in dosages up to 100 grams over a period of 20 days in a case of beryllium poisoning the calcium Versenate was without effect on the poisoning but that might have been anticipated There were no indications of any kind of toxicity from the chelating agent One would anticipate this lack of toxicity from the recent data on the clearance and distribution of the radioactively labeled material

Butler Isn t there an investigator in Framingham Massachusetts who has worked extensively with the agents?

Rubin Yes Dr F C Bersworth

Sobel Do you not predict that there will be a magnesium deficiency as a con equence of the continuous administration of the calcium salts?

Rubin In other words you are suggesting that simultaneously with the excretion of calcium there may be a magnesium loss?

Sobel Yes Is there an exchange to magnesium?

Rubin No We have done *in vitro* studies in which we have shown that magnesium is not bound in the presence of calcium The point is that the differential in the equilibrium constant is roughly of the order of 100 to 1 which means that none of the magnesium is bound when there is calcium present to be taken up as is always the case *in vivo*

Shorr Do you know what Versene does to tissues *in vivo*? I am wondering about the possibility of using it for rachitic cartilage studies and whether one could remove calcium from compact bone without damaging the preparation

Rubin Well Versene is being u ed to decalcify bones and teeth as we have pointed out

Shorr I was wondering whether the chelating agent damages living structures

Rubin The morphology after Versene treatment appears to be excellent The compound is being used as a matter of fact in a procedure for counting platelets because it gives a unique preservation of morphologic and staining characteristics

Shorr Are the platelets fixed or living?

Rubin They are living

Neuman After Versene?

Rubin The counting is done on drawn blood

Handler How much Versene was used?

Rubin I cannot answer that question

Neuman If you are decalcifying you will kill all of the cell

Rubin The counting of platelets is done in whole blood

Handler Versene will prevent the blood from clotting

Rubin Yes 1 mg per cc is all that is needed

Handler And the red cells will not glycolyze at all

Armstrong Let us return to Dr Shorr s question Can a bone decal cified with Versene be made to recalcify *in vitro*?

Rubin I do not know

Neuman We have done a little work with Versene and we certainly have been able to find all kinds of alterations after it has been used The cells are dead and my guess at the moment is that recalcification will not occur

Handler Versene in reasonable quantity is an excellent inhibitor of glycolysis in any system I do not think that this inhibition is very readily reversed

Shorr Versene has considerable usefulness if it can be employed in amounts low enough to eliminate damage to the cells What concentrations of it will inhibit glycolysis?

Handler I do not remember but I have the figures and can send them to you

Urist Does Versene produce a lesion in the skeleton that in any way resembles that found in the Fanconi syndrome?

Rubin I cannot answer that

Kramer What Fanconi syndrome?

Follis Versene does produce excessive destruction very soon That is all I know about it

Sobel The Fanconi syndrome is essentially a loss of phosphate rather than of calcium

Follis The destruction would depend on how much of it you give for how long a period of time

CLOSING REMARKS

Armstrong I think it is appropriate that we end the Conference on this very interesting note Before we close I wish to make certain acknowl edgements

First I would like to say to Professor McCance how much we appre ciate his having been here and how much we have profited from his visit to this Conference We know that the impact of your visit to this country has been even broader than that which you brought here to the Conference

because you have been able to visit several centers of research in the United States We wish only that you could stay longer but we realize that the sailing of *The Queen Mary* is not entirely under your control!

I want especially to thank our other guests who have contributed so much to the Conference I should like also to thank the members who have attended all five Conferences especially those who have undertaken the very difficult and arduous task of preparing the introductory presentations

I am going to single out only two of these persons for special mention First Dr Reifenstein who has served so capably as the Editor of our TRANSACTIONS I know that the labor he has put into this task has been tremendous I do not understand how he can spare the time required to do the work in such a capable and thorough fashion [Applause]

The second member to whom I wish to give special thanks is Dr Ephraim Shorr who has been our house doctor [Laughter] He has had the problem of taking care of me when I had renal colic and just last night he had to treat Dr Copp s fracture—both cases you see relating to calcification processes

Shorr A physician has to keep in practice you know

Hodge Mr Chairman may I speak not to you but to our host for just a moment? Dr Fremont Smith as a member of the fifth Conference and of some of the others and also because I believe I can speak for the guests I would like to tell you how much we have enjoyed and appreciated the expert chairmanship of Dr Armstrong [Applause]

Sobel And may I say as a constant guest for the last four years that while we cannot pay in currency for the privilege of attending this Conference we can pay in a coin that we all can give to both Dr Fremont Smith and Dr Armstrong namely by a rising vote of thanks for conducting these Conferences so well and for making us all feel so much at ease

[The members and guests arose and applauded]

Fremont Smith May I thank you all very much indeed and especially our Chairman and Dr Reifenstein for the noble work he has done and will do as Editor

Armstrong Ladies and Gentlemen the Conference on Metabolic Interrelations has adjourned *sine die*

— Adjournment —

METABOLIC INTERRELATIONS

Cumulative Index

1949 1953